Cleveland Clinic Quarterly

Volume 27 JULY 1960 No. 3

HYPOGASTRIC ARTERIOGRAPHY PRIOR TO CONTINUOUS INFUSION OF MALIGNANT TUMORS OF THE UTERINE CERVIX AND VAGINA

A Preliminary Report

PAUL R. ZEIT, M.D.,
Department of Obstetrics and Gynecology
C. ROBERT HUGHES, M.D.,
Department of Radiology
JOHN J. CAHILL, M.D.,* and J. GREGORY HAMILTON, M.D.*
Department of Obstetrics and Gynecology

RECENTLY, Sullivan, Miller, and Sikes[1] reported the treatment of advanced squamous-cell carcinoma of the mouth and the nasopharynx by continuous intraarterial infusion with amethopterin. In several of their patients regression of the tumor was so striking that an attempt to adapt their technics and methods to the treatment of squamous-cell carcinoma of the uterine cervix and the vagina was undertaken here at the Cleveland Clinic. The project has been in progress since December, 1959, and this preliminary report is concerned with the visualization of the vascular anatomy of the uterus and of the upper vagina in nine patients.

For maximum benefit from infusion of malignant tumors in any location, the following four requirements must be met: (1) the one or two arteries selected for infusion must deliver most of their blood to the region of the tumor; (2) most of the blood supply of the tumor itself must come from these arteries; (3)

*Fellow in the Department of Obstetrics and Gynecology.

the vessels must be accessible and of adequate size to be catheterized easily; (4) the sacrifice of the arteries must be possible, if necessary, without serious damage to normal tissues. For infusion of carcinoma of the uterus, cervix, and upper vagina, these four requirements are fulfilled by employing the hypogastric arteries. Sullivan and Miller[2] have used these arteries for the infusion of tumors of the bladder in two male patients. It is logical that the hypogastric arteries be used in female patients for treatment of carcinoma of the cervix and of the vagina.

We have followed a strict policy of performing arteriography at the time of laparotomy on every patient who is to receive hypogastric arterial infusion. From the arteriogram, information is obtained concerning the accuracy of placement of the catheter in the artery, and an accurate estimation is possible of the amount of the drug that will reach the region of the tumor. We have observed no undesirable side effects from the contrast medium in any of our patients, nor any complications from the procedure of arteriography.

Anatomy of the Hypogastric Arteries

The many variations of the hypogastric arteries have been noted by anatomists and surgeons for many years. A classic description and tabulation of the variations was given by Lipshutz[3] in 1918, and this has been confirmed and enlarged upon by many others, among whom are Quimby,[4] and Shafiroff, Grillo, and Baron.[5] In general, the arterial branches are anatomically classified as parietal and visceral groups, and it is the visceral group that is used in the chemotherapy of carcinoma of the cervix and of the vagina.

Figure 1 shows an arteriogram of a normal right hypogastric artery in an adult female. It demonstrates the division of the artery, soon after its origin, into an anterior and a posterior trunk. This early division is an almost constant feature of the artery, but there is individual variation in the branches derived from each trunk. The superior gluteal artery always arises from the posterior trunk, and in the arteriogram shown it is the only major branch (C) of this trunk; this is considered the most common arrangement.[5] A second frequent pattern is that demonstrated in *Figure 2,* where both the superior and the inferior gluteal arteries arise from the posterior trunk. The left posterior trunk has been ligated so that neither gluteal artery contains contrast medium. A catheter lies in the right posterior trunk, and on this side the gluteal arteries are the only arteries shown (A and B).

In six of the nine patients the more common anatomic arrangement was found, wherein the superior gluteal artery arose from the posterior trunk of the hypogastric artery, and the inferior gluteal artery arose separately from the hypogastric artery. In these patients the posterior trunk and the inferior gluteal artery had to be ligated separately, to divert to the visceral branches most of the blood flow in the hypogastric artery. In the remaining three patients the posterior trunks gave rise to both the superior and inferior gluteal arteries; in each of these patients

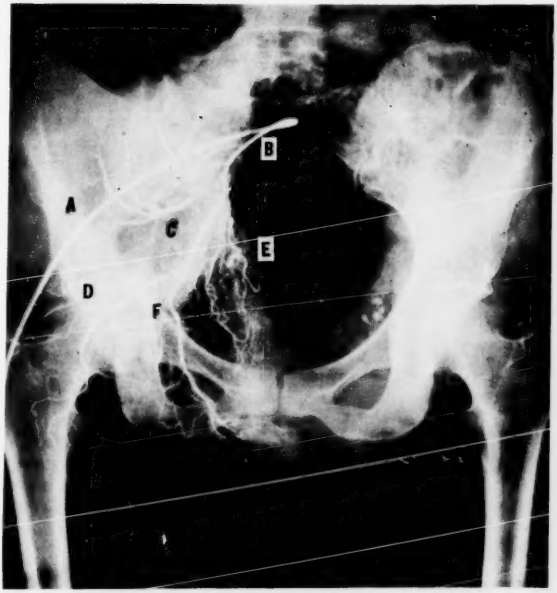

Fig. 1. Right hypogastric arteriogram showing the most common variant of the hypogastric artery in an adult female: A, Catheter leading into hypogastric artery near its origin. B, Hypogastric artery before its bifurcation. C, Superior gluteal artery, which in this patient is the only branch of the posterior trunk. D, Inferior gluteal artery. E, Uterine artery. F, Internal pudendal artery.

only one ligature was needed. Thus, ligation of the posterior trunk prevents flow of the infusate into the superior gluteal artery in almost every instance, and into the inferior gluteal artery, as well, in many patients. Elimination of blood flow in these two vessels directs almost all of the flow toward the uterus, cervix, and vagina, through the uterine and the internal pudendal arteries *(Figs. 2 and 3)*. In *Figure 3* can be seen an ideal distribution of flow for infusion of tumors of the cervix and the vagina, which meets the first requirement in effective infusions.

The second requirement for effective infusions is that most of the blood supply of the tumor be derived from the arteries that are to be infused. The uterus and the cervix receive their blood supply from the uterine and ovarian arteries; the vagina is supplied by the vaginal, the middle hemorrhoidal, and the internal pudendal arteries, all of which are branches of the hypogastric arteries.

The size and accessibility of the hypogastric arteries are well known to all pelvic surgeons. The hypogastric arteries can be easily exposed with a minimum of dissection during the exploratory laparotomy that always should be performed to determine the extent and the distribution of the neoplastic tissue. Both hypo-

gastric arteries can be ligated at their origins without damage to the rectum, anus, bladder, or perineum.[4-8] It has been our experience that ligation of both hypogastric arteries and both ovarian arteries produces no ill effects.

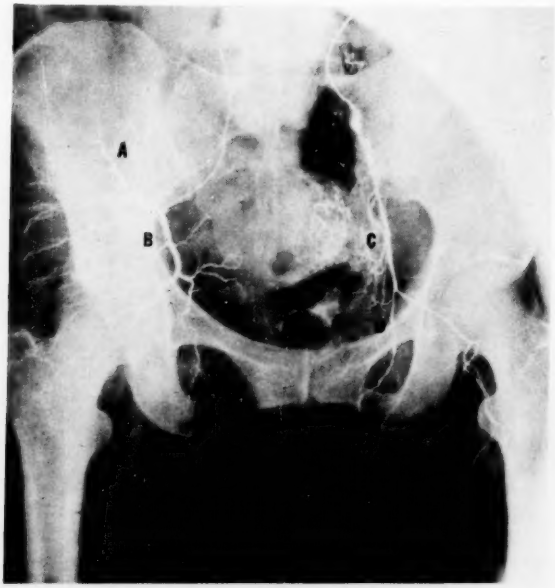

Fig. 2. Bilateral arteriogram showing the second most common variant of the hypogastric artery in an adult female. The superior and inferior gluteal arteries both arise from the posterior trunk. On the patient's right only the posterior trunk and its branches are filled with contrast medium. On her left only the anterior trunk and its branches are visualized. A, Superior gluteal artery (right). B, Inferior gluteal artery (right). C, Uterine artery (left). D, Internal pudendal artery (left).

Technic of Arteriography

A hypogastric artery is exposed by reflecting the peritoneum. First the posterior trunk is ligated with a silk ligature as close to its origin as possible. This is done before the catheter is threaded into the vessel, because the posterior trunk lies in almost a direct line with the first part of the hypogastric artery, and the catheter will usually thread into the posterior trunk. Two bulldog clamps are placed on the artery, one clamp near its origin from the common iliac artery, and the other clamp well down on the anterior trunk. Between the two clamps a No. 18 needle is inserted through the wall of the vessel, and through the lumen of this needle a fine polyethylene catheter is threaded. The needle is withdrawn over the catheter, and the bulldog clamps are released. Catheterization is an almost

bloodless procedure; the elasticity of the vascular wall is usually sufficient to prevent leakage around the catheter. From 7 to 8 ml. of Hypaque Sodium 50%* is injected rapidly through the catheter, and as the injection is completed the roentgen film is exposed.

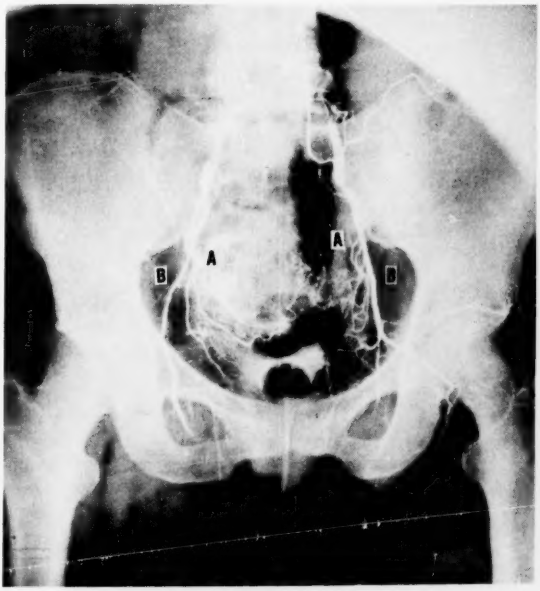

Fig. 3. Bilateral arteriogram (same patient as *Figure 2)* after both posterior trunks were ligated. The elimination of the gluteal arteries from the hypogastric circulation has directed most of the flow to the region of the uterus, cervix, and vagina. Note also the evidence of the concentration of contrast medium in the region of the bladder. A, Uterine artery. B, Internal pudendal artery.

Summary

Four anatomic requirements for a technically successful continuous infusion of a malignant tumor in any part of the body are: (1) the one or two arteries selected for infusion must deliver most of their blood to the region of the tumor; (2) most of the blood supply of the tumor itself must come from these arteries; (3) the vessels must be accessible and of adequate size to be catheterized easily; (4) sacrifice of the arteries must be possible, if necessary, without serious damage to normal tissues.

The anatomic arrangement of the hypogastric arteries in the human female, as demonstrated by arteriograms, is such that the above four requirements can be fulfilled for infusion of malignant tumors of the uterine cervix and vagina.

**Winthrop Laboratories.*

Ligation of the superior and inferior gluteal arteries diverts most of the blood flow in the hypogastric artery to the uterine, internal pudendal, middle hemorrhoidal, and vaginal arteries.

The technic of hypogastric arteriography has been briefly described. In the nine patients reported by the authors there were no undesirable effects or complications arising from the procedure.

References

1. Sullivan, R. D.; Miller, E., and Sikes, M. P.: Antimetabolite-metabolite combination cancer chemotherapy. Effects of intraarterial Methotrexate-intramuscular citrovorum factor therapy in human cancer. Cancer **12:** 1248-1262, 1959.

2. Sullivan, R. D., and Miller, E.: Personal communication, May 12, 1960.

3. Lipshutz, B.: Composite study of hypogastric artery and its branches. Ann. Surg. **67:** 584, 1918.

4. Quimby, W. C., Jr.: The Anatomy and Blood Vessels of the Pelvis, Chap. 1, pp. 26-64, *in* Surgical Treatment of Cancer of the Cervix, edited by J. V. Meigs. New York: Grune & Stratton, Inc., 1954, 453 pp.

5. Shafiroff, B. G. P.; Grillo, E. B., and Baron, H.: Bilateral ligation of hypogastric arteries. Am. J. Surg. **98:** 34-40, 1959.

6. Daro, A. F.; Nora, E. G., Jr.; Gollin, H. A., and Howell, R. E.: Artery ligation in bleeding cervical cancer. Am. J. Obst. & Gynec. **78:** 197-199, 1959.

7. Decker, W. H.: Ligation of hypogastric artery in postoperative uterine artery hemorrhage; report of case. Obst. & Gynec. **5:** 109-110, 1955.

8. Hecht, E. L., and Blumenthal, E. D.: Recurrent bleeding from vaginal vault following total abdominal hysterectomy. Am. J. Obst. & Gynec. **67:** 195-197, 1954.

THE PRESERVATION OF THE COAGULATION SYSTEM IN STORED WHOLE BLOOD

DONALD A. SENHAUSER, M.D.
Department of Clinical Pathology

THE problem of hemorrhagic diathesis during massive transfusion therapy has been recognized for several years.[1-4] The studies by Stefanini and Chatterjea,[1] Krevans and Jackson,[3] and Jackson, Krevans, and Conley[4] indicated that this phenomenon is dependent on the rapid infusion of large amounts of platelet-poor whole blood. Stefanini, Mednicoff, Salomon, and Campbell[2] demonstrated that the abnormal bleeding could be corrected by the immediate infusion of fresh blood platelet concentrates or by closely repeated direct transfusions of fresh whole blood.

Procedures that require large amounts of whole blood, such as open cardiotomy, dialysis, and arterial grafting, have been widely employed at the Cleveland Clinic Hospital for several years. Hemorrhagic diathesis has rarely occurred, because all blood is drawn immediately prior to such scheduled procedures. However, in certain emergency procedures, it was necessary to utilize large quantities of banked blood immediately at hand. In several instances, a hemorrhagic syndrome ensued, similar to the cases reported by Stefanini and associates[2], which were characterized by low platelet counts, increased capillary fragility, prolonged bleeding time, prolonged clotting time, and impaired prothrombin consumption. In our cases, the surgical hemorrhage was controlled by utilizing direct blood transfusions.

As the number of such emergency procedures increased, it became apparent that it would be impossible to meet the increased demand for fresh whole blood at any time of the day or night. The urgent problem was to devise a method of administering large volumes of blood of varied storage periods in a manner that would obviate the hemorrhagic syndrome. In order to do so, it was first necessary to determine the length of time the coagulation mechanism would remain intact in blood taken into nonvacuum siliconized bottles of anticoagulant acid citrate dextrose solution (ACD) using nonwettable donor sets. At the time the investigation was undertaken, the published studies on this problem seemed inapplicable to our situation.

Tests that were developed during the recent past to study blood coagulation defects were adapted and modified for the present study to analyze the coagulation defects in stored whole blood. From this information, it was hoped to determine the storage stability of blood as it is processed in the Cleveland Clinic blood bank, and to devise a satisfactory plan for administering massive transfusion therapy.

For purposes of analysis, the coagulation mechanism can be considered as occurring in three sequential stages: stage I, evolution of thromboplastin; stage II,

This paper is an expansion of an original thesis by Doctor Senhauser, which won The William E. Lower Fellowship Prize Award, for the year 1959, sponsored by The Frank E. Bunts Educational Institute.

conversion of prothrombin to thrombin by thromboplastin; and stage III, conversion of fibrinogen to fibrin by thrombin. *Table 1* lists the factors known to play a role in each of these stages of coagulation.

Table 1.—*Blood factors in the three stages of coagulation*

Source		
Stage I		
Blood platelets *plus* Antihemophilic-globulin (AHG) Plasma thromboplastin component (PTC) Plasma thromboplastin antecedent (PTA) Hageman factor Stuart factor Factor V	Ca^{++} Wettable surface →	Thromboplastin
Stage II		
Prothrombin	Thromboplastin *plus* V, VII, Stuart, Ca^{++} →	Thrombin
Stage III		
Fibrinogen	Thrombin, Ca^{++} →	Fibrin

The factors that are known to be the most labile during storage are the platelets, antihemophilic-globulin (AHG), and factor V (Ac-globulin, proaccelerin, or labile factor). Hence, the primary concern was the fate of these three factors in banked blood.

Materials

The blood bank of the Cleveland Clinic utilizes three sources for the supply of blood for transfusion. The primary source is the American Red Cross Regional Center; blood from this center is drawn into nonsiliconized vacuum bottles. The second source is the professional donor, and the "repay" donor, whose blood is drawn and is processed at the Clinic blood bank. All such donor blood is collected in siliconized nonvacuum glass bottles, with nonwettable donor sets, utilizing only gravity flow during phlebotomy. The third source is the commercial blood banks of the area. Blood at these banks is drawn into nonsiliconized partial-

vacuum bottles. All three types of containers utilize the standard ACD solution as an anticoagulant preservative. All blood is stored at 4 C. until it is removed just prior to use.

Whole blood samples. Samples for testing were obtained from two sources: random bottles were sampled just prior to distribution from the blood bank; and serial samples were obtained from donor blood drawn especially for this project. All such blood was kept at 4 C. for the duration of the testing period, except during the time necessary to obtain samples for testing. Blood samples were obtained with siliconized syringes and needles; these samples were placed in siliconized glass tubes for further processing.

Low-spun (platelet-rich) plasma. Platelet-rich plasma was always obtained by spinning the whole blood samples in siliconized tubes at 500 rpm for 10 minutes in the Serval Angle table-model centrifuge with a 27-place head.

High-spun (platelet-poor) plasma. Platelet-poor plasma was obtained by spinning platelet-rich plasma in siliconized tubes at 3000 rpm for 20 minutes in the Serval Angle centrifuge.

Platelet suspension. Platelet suspensions were prepared by washing the sediment obtained from high-spun plasma twice in 0.85 per cent saline solution, and, after the final washing, thoroughly resuspending the platelets by vigorous agitation with a wooden applicator stick. The final volume of saline solution was one third the original volume of platelet-rich plasma.

Methods

Platelet count. All platelet counts were made on samples of whole blood after a standard two-minute mixing period. The direct method of Rees-Ecker, as described by Wintrobe,[5] was used. The counts were made in duplicate by one person* in order to standardize the method as much as possible. The direct method was chosen as a result of the report of Wilson, Eisemann, and Chance,[6] which indicated that the direct method of platelet counting was unreliable when applied to their similar studies. Platelet morphology was studied on all samples from blood smears made on coverslips and stained with Wright's stain. All smears were evaluated by one observer (D.A.S.).

Thromboplastin generation test (TGT). The thromboplastin generation tests were performed according to the method of Biggs and Douglas,[7] utilizing a more concentrated platelet suspension, as described above. It was determined by a series of pilot experiments that the use of ACD solution, as the anticoagulant, did not affect the test. In addition, several modifications of the original method were developed in order to test for thromboplastic activity in the stored plasma. These are described later in this report.

Recalcified clotting time. To 0.1 ml. of platelet-rich plasma was added 0.28 per cent of calcium chloride ($CaCl_2$), and the mixture was incubated in a 37 C. water

**Miss Mary Margaret Potter, B.A., M.T. (A.S.C.P.), made all the counts, and rendered other invaluable technical assistance throughout this study.*

bath. After the first minute of incubation, the tube was tipped every 15 seconds until the fibrin clot appeared. Each test was run in duplicate or triplicate, with fresh low-spun plasma as a control.

Clot retraction, percentage. The clot retraction was determined by placing a measured amount of low-spun (platelet-rich) plasma into a 12-mm. conical, graduated, Pyrex centrifuge tube. A volume of 2.5 per cent $CaCl_2$ equal to one tenth the volume of plasma was added, and a wooden applicator stick was inserted into the tube. The mixture was incubated for one hour in a 37 C. water bath. The wooden applicator stick and adherent clot were then removed; the remaining serum-$CaCl_2$ mixture was measured, and is expressed as the percentage of the original volume. For each test, a similar sample of fresh low-spun plasma was used as a control system.

Prothrombin time. The one-stage prothrombin time was determined by the standard method of Quick,[8] using a commercial preparation of brain thromboplastin. In the correction studies, barium sulfate ($BaSO_4$)-adsorbed, fresh-frozen, Seitz-filtered beef plasma, as well as a commercial preparation, was used as the source of factor V. These methods are qualitative; no quantitative measurements were attempted for assay of prothrombin or factor V.

Russell's viper venom (Stypven) coagulation time.* Stypven is a powerful anticoagulant that accelerates the conversion of prothrombin to thrombin in the presence of a platelet lipoid factor and calcium ions. Its action is independent of factor VII, thereby differing from brain thromboplastin[9]. Stypven coagulation time was determined using a procedure based on O'Brien's[10] method and modified for the present study. One tenth of one milliliter of 0.28 per cent $CaCl_2$ was added to an incubation tube in a 37 C. water bath, which contained 0.1 ml. of the test plasma, 0.1 ml. of platelet suspension, and 0.1 ml. of 1:10,000 Stypven. The clotting time of the resultant mixture is recorded in seconds. One-tenth milliliter of 0.85 per cent saline solution was substituted for the platelet suspension in the incubation tube, when necessary, to keep the total volume constant.

Results

Platelet count. Platelet counts were performed on random samples obtained from 60 nonvacuum siliconized bottles stored in the blood bank. Almost all of these bottles contained 200,000 platelets per cubic millimeter during the first three days of storage. The number of platelets declined in gradual fashion until the seventh day, when most bottles contained about 150,000 platelets per cubic millimeter; after this time there was a rapid and erratic decrease in platelet numbers in the stored blood.

The curves in *Figure 1* represent the combined results of serial platelet counts on stored blood obtained by two methods of phlebotomy. The platelet counts of the blood taken into vacuum nonsiliconized bottles showed a notable platelet loss immediately after phlebotomy. In these bottles, the number of platelets

**Burroughs Wellcome & Co. (U.S.A.) Inc.*

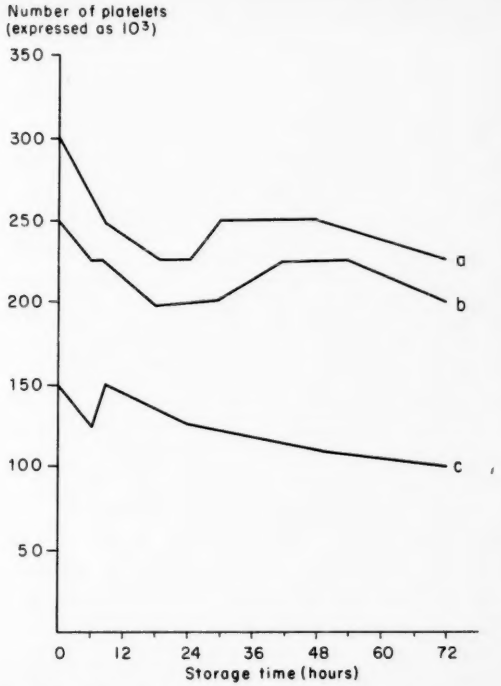

Fig. 1. Graph showing serial platelet counts of blood stored in various containers: a and b, range of serial platelet counts in several nonvacuum siliconized bottles; c, average of serial platelet counts in several vacuum nonsiliconized bottles.

decreased rapidly during the first three days of storage. By contrast, blood taken into nonvacuum siliconized bottles showed little or no immediate decrease in number of platelets, and the platelet counts declined only slightly during three days of storage. The serial nature of these determinations, each necessitating a period of mixing, probably resulted in values lower than if the bottles had been undisturbed. With each mixing there was unavoidable foaming, which is a major factor in platelet destruction.[11] These results are in good agreement with the report of Mustard and Walker,[12] who utilized similar technics.

Clot retraction, percentage. In control experiments using fresh low-spun plasma, clot retraction began promptly and was complete at the end of one hour. The fibrin clot was tightly adherent to the wooden applicator stick, and virtually no serum remained entrapped. The clot was firm, rubbery, and glistening white. As the plasma aged, especially after 48 hours, the speed of retraction decreased, and retraction became variably incomplete. The fibrin clot became soft, gelatinous, and grayish-white.

Clot retraction was universally good during the first 48 hours of storage in nonvacuum siliconized bottles. After this time, in many bottles there was a rapid and erratic decline in clot retraction, although in many bottles tested it was still 80 per cent complete at 72 hours. After 72 hours, the speed and effectiveness of clot retraction declined rapidly.

In nonsiliconized vacuum bottles, there was great variability in clot retraction from sample to sample of blood stored for the same period of time. In general, the clot retraction was much slower and incomplete in such samples, and it was not possible to predict the efficacy of clot retraction in the various samples of the same age stored in this type of container.

Platelet morphology. The platelets passed through several definite morphologic phases during storage. The times at which these changes were noted varied from sample to sample, but were consistently present in each.

During the first four to six hours, the platelets resembled those seen in a fresh direct preparation. From 6 to 18 hours, they appeared swollen, with rounded or discoid forms predominating; the hyalomeres were granular and of the usual size and color. After 18 hours, the hyaloplasm of increasing numbers of platelets had pseudopodia or mycelial-like projections. Clumping was a prominent feature during this period. Few rounded forms remained at 24 to 30 hours, and increasing numbers of platelets appeared which consisted of a dark, basophilic, finely granular hyalomere surrounded by scanty amounts of basophilic cytoplasm. During the next 18 hours, these forms became increasingly numerous, until at 48 hours, and thereafter, the dominant form was the small basophilic hyalomere with scanty, deeply basophilic cytoplasm. This platelet form gave the smears a characteristic "peppered" appearance when viewed at a magnification of 430 x.

Thromboplastin generation test. The results of the TGT are summarized in *Table 2*. The thromboplastic activity of the platelets collected and stored in nonvacuum siliconized bottles remained entirely normal up to 72 hours, when tested with normal $BaSO_4$-adsorbed plasma and normal serum. It then decreased slightly, but the platelet suspensions remained remarkably active up to seven days.

Qualitative assays of AHG were also determined. There was a rapid decrease in AHG activity in the stored plasma, which was rather notable by 24 hours, when tested with normal platelets and normal serum. AHG activity declined only slightly thereafter.

Thromboplastin generation improved when stored platelets and stored plasma were tested together. This observation, in addition to the apparent thromboplastic activity of AHG-deficient 48-hour plasma, seemed to indicate the presence of a thromboplastic factor in the plasma itself. Stefanini[13] had previously noted this effect. As shown in *Table 2*, stored high-spun (platelet-poor) $BaSO_4$-adsorbed plasma and normal serum were used alone as a source of thromboplastin. The 48-hour, 72-hour, and 168-hour-old plasma showed remarkable thromboplastic activity. In

contrast, fresh plasma that was similarly treated showed practically no such activity.

Table 2.—*Results of thromboplastin generation tests (TGT) on stored whole blood in nonvacuum siliconized bottles*

Incubation mixtures	Substrate clotting time, seconds						
	Intervals of incubation, minutes						
	One	Two	Three	Four	Five	Six	Seven
Normal plasma, serum, platelets	22″	7″	7″	7″	—	—	—
Normal plasma, serum, 1-hr. platelets	25	8	7	7	7	—	—
Normal plasma, serum, 4-hr. platelets	10	7	7	7	—	—	—
Normal plasma, serum, 24-hr. platelets	17	8	8	8	8	—	—
Normal plasma, serum, 48-hr. platelets	—	7	7	7.5	—	—	—
Normal plasma, serum, 72-hr. platelets	45	14	10	10	10.5	—	—
1-hr. plasma, normal serum, platelets	25	8	7	7	7	—	—
4-hr. plasma, normal serum, platelets	19	8	9	8.5	9	—	—
24-hr. plasma, normal serum, platelets	44	12.5	12.5	11	10	—	—
48-hr. plasma, normal serum, platelets	—	9	9	8.5	9.5	—	—
72-hr. plasma, normal serum, platelets	—	26	10	10	9.5	10	—
24-hr. plasma, normal serum, 24-hr. platelets	29	11	10	9	—	—	—
48-hr. plasma, normal serum, 48-hr. platelets	—	9.5	8	8	—	—	—
72-hr. plasma, normal serum, 72-hr. platelets	41	17	9.5	11.5	12	—	—
$BaSO_4$-high-spun plasma, normal serum and saline solution	>90	>90	>90	>90	75	58	—
$BaSO_4$-high-spun 48-hr. plasma, normal serum, saline solution	>90	17.5	16	16	16.5	—	—
$BaSO_4$-high-spun 72-hr. plasma, normal serum, saline solution	>90	88	59	35	25	18	—
$BaSO_4$-high-spun 168-hr. plasma, normal serum, saline solution	>90	>90	>90	45	17	—	—

Stypven coagulation time. *Table 3* summarizes data of a typical mixing experiment utilizing the Stypven coagulation time. The Stypven coagulation time was 18 seconds for fresh low-spun (platelet-rich) plasma. When the same plasma was high-spun, and the platelet-poor sample was tested, the Stypven coagulation time had lengthened to 25 seconds. When the thrice-washed platelet suspension was then returned to the plasma, the clotting time was shortened to 11 or 12

Table 3.—*Results of mixing experiments using Stypven coagulation time**

Fresh plasma, ml.			19-hr. plasma,† ml.			3-day plasma,† ml.			Stypven time, seconds
Low-spun	High-spun	Platelets‡	Low-spun	High-spun	Platelets‡	Low-spun	High-spun	Platelets‡	
0.1	—	—	—	—	—	—	—	—	18
—	0.1	—	—	—	—	—	—	—	25
—	0.1	0.1	—	—	—	—	—	—	11
—	—	—	0.1	—	—	—	—	—	13
—	—	—	—	0.1	—	—	—	—	13
—	—	—	—	0.1	0.1	—	—	—	9.5
—	—	—	—	—	—	0.1	—	—	13.5
—	—	—	—	—	—	—	0.1	—	14
—	—	—	—	—	—	—	0.1	0.1	9
—	0.1	—	—	—	0.1	—	—	—	10
—	0.1	—	—	—	—	—	—	0.1	9.5
—	0.1	—	—	—	—	—	0.1	—	15
—	—	—	—	—	—	—	0.1	0.1†	9.5
—	—	—	—	—	—	—	0.1†	0.1	8.0
—	—	0.1	—	—	—	—	0.1	—	10

*The methods of preparing the plasma and platelet suspensions are given in the text, page 127. In each case, 0.1 ml. of 1:10,000 Stypven and 0.1 ml. of $CaCl_2$ were added to the reaction mixture.

†These samples were from whole blood that was stored at 4 C. for the time indicated.

‡When platelet suspensions were omitted, 0.1 ml. of 0.85 per cent saline solution was added to the reaction mixture to make up the volume.

seconds. In contrast, using plasma samples from stored blood, there was no difference in the Stypven coagulation times of platelet-rich and of platelet-poor plasma. When the suspensions of stored platelets were returned to their respective stored plasmas, in each case the Stypven coagulation time of the mixture was again shortened. The activity of stored platelets as measured by adding suspensions of stored platelets to fresh high-spun plasma, was equal to or greater than the fresh-platelet suspensions. Of great significance is the observation that when high-spun, three-day old plasma was used as a source of platelet factor, using high-spun fresh plasma as the test sample, the Stypven coagulation time was shortened from 25 seconds to 15 seconds.

From the results of this series of experiments, one may state that: (1) the platelet factor necessary to produce plasma coagulation with Stypven is present and active in stored as well as in fresh platelets; (2) the processing of platelets (e.g., making platelet suspensions) in such a fashion as to cause lysis and disinte-

gration, accelerates the conversion of prothrombin to thrombin by Stypven; and (3) the platelet factor remains active after diffusion into the plasma, and appears to be storage stable in such plasma, at least as long as 72 hours at 4 C.

Prothrombin time. The prothrombin time was normal (14 seconds) in all samples during the first 12 hours of storage. After this time, there was an abrupt prolongation of the prothrombin time in more than one half of the samples tested. By the end of 24 hours, all samples showed prolonged prothrombin time. This prolongation continued in a gradually progressive fashion through 96 hours. Thereafter, the prothrombin time stabilized, and in all samples tested between the fifth and eleventh days of storage the range of prothrombin time was 18 to 20 seconds.

During the first 96 hours, the addition of 1 part of factor V to 10 parts of stored plasma, returned the prothrombin time to 14 seconds. The addition of factor V to 5-to-14 day old plasma shortened the prothrombin time to less than 15 seconds in all cases.

Recalcified clotting time. Twenty of thirty plasma samples had normal recalcified clotting times during the first 24 hours of storage. Only one sample had a clotting time longer than 130 seconds during the first 12 hours, and four samples were longer than 130 seconds during the second 12 hours. Of 42 plasma samples, 29 had a clotting time within normal limits during the first 72 hours, and of the 13 in which it was longer than 120 seconds, in only five was it longer than 130 seconds. In each case, the prolonged clotting time could be corrected by the addition of factor V during the first 48 hours of storage.

In general, the over-all clotting mechanism of the stored plasma was intact, as measured by this in vitro test. This was true despite demonstrable deficiencies of one part or another of the coagulation mechanism.

Discussion

Since Lewisohn[14] described a procedure to preserve blood for transfusion in sodium citrate solution, efforts have been made to prolong the period during which blood could be safely stored. Most of this work has emphasized the preservation of the erythrocytes during storage. The occurrence of hemorrhagic diathesis during or immediately after the use of banked blood for massive transfusion therapy resulted in increased interest in the effect of storage on the coagulation system of drawn blood.

Thrombocytopenia after multiple transfusions of banked blood has been well demonstrated.[1-4] A great decrease in the number of platelets usually occurs only after a massive transfusion (5000 ml. or more in adults).[3] The occurrence of hemorrhagic diathesis has been related directly to the thrombocytopenia, with relatively minor changes noted in amounts of factor V and AHG in normal persons.[1] Finkbiner, McGovern, Goldstein, and Bunker[15] have recently shown that those two factors may be of importance in patients with severe hepatic disease.

In the past, it has been observed that other factors influence the platelet count in patients who have had severe hemorrhage, or after surgical procedures.[16] Desforges, Bigelow, and Chalmers[17] studied the decrease in number of circulating platelets during gastrointestinal hemorrhage, and concluded that the decrease was not related to the amount of blood loss, or to the presence or absence of shock. Warren, Lauridsen, and Belko [18] postulated that the stress of major surgery, acting through the adrenal cortex, caused the decrease in platelets in their patients. They concluded that this decrease was independent of such factors as severity of hemorrhage, transfusion, or hemodilution. They could produce the same effect through administration of corticotropin.

However, the effect of surgical trauma and stress on platelet count seems to have been unclear in the past. The division of opinion is noted in a review of the subject by Mustard.[19] In his own series of 21 nontransfused patients who underwent surgery, he noted about a 10 per cent increase in the platelet count in the immediately postoperative period. In contrast, in 62 patients who underwent surgical procedures and who received transfusions, Mustard[20] found that one half demonstrated a decrease in circulating-platelet count. Similarly, Stefanini and Chatterjea,[1] noted that of 36 patients receiving platelet-poor whole blood from normal donors, 32 showed a transient but significant thrombocytopenia. Krevans and Jackson[3] reported on 27 adults, 14 of whom received more than 5000 ml. of stored blood, and in all of them thrombocytopenia developed; 11 of them had clinically abnormal bleeding. In the other 13, who received less than 5000 ml. of stored blood, thrombocytopenia developed in a few, but none of these had abnormal bleeding.

In the light of these reports, it would seem that the primary cause of hemorrhagic diathesis in massive transfusion therapy is the transfusion of stored whole blood, and that stress, adrenocortical hormones, and shock, play secondary roles in this syndrome.

The clotting mechanism in vitro. I. *Platelets. Platelet counts.* The studies presented in this report illustrate that blood drawn into nonvacuum siliconized bottles, utilizing nonwettable donor sets, generally shows a high platelet count immediately after phlebotomy, in contrast to the counts for the other methods tested. Further, the decline in platelet numbers during storage was slower and more gradual in nonvacuum siliconized bottles than in nonsiliconized vacuum bottles. These results are in accord with those of Mustard and Walker,[12] who showed that platelet preservation during storage is dependent upon the numbers of platelets lost during phlebotomy. The probable mechanism of this rapid decrease in platelet numbers is the activation of thrombin that, in turn, acts as a catalyst on the platelets, causing increased lysis of those remaining after phlebotomy. After these results had been determined, further tests of the clotting mechanism were largely confined to blood stored in siliconized nonvacuum bottles.

Platelet survival. Platelet survival can be estimated in a variety of ways, accord-

ing to: platelet morphology, thromboplastic activity, and clot retraction.

Platelet morphology. There seems to be little agreement in the literature regarding correlation of form and function of platelets, despite recent advances in electron and phase microscopy.[21-24] However, there is general agreement that the normal shape is discoid, and this proved to be true in this study with the light microscope. The progressive morphologic changes that were observed, were the same as those reported by others,[23] although the speed of change varied with the technics used. Despite the apparent regularity of the changes noted in the stored platelets, it was not possible to judge accurately their activity or viability from these morphologic forms. Tullis[21] stated that intactness does not imply normal survival.

Thromboplastic activity. The TGT can be used to measure relative or qualitative thromboplastic activity, although it is not satisfactory as a quantitative test. The results here reported show that storage has little effect on the thromboplastic activity of the platelets. Mustard,[23] Tullis,[21] and Minor and Burnett[25] found that platelets stored by various methods retained their ability to assist in the generation of thromboplastin in vitro.

The $BaSO_4$-adsorbed high-spun plasma obtained from stored whole blood had a distinct ability to generate thromboplastin when used as a platelet substitute in the TGT. This confirms the observation of Stefanini and co-workers[11,26] that the hemostatic effect of platelets must be due, at least in part, to certain relatively stable constituents that are liberated when these bodies disintegrate. They further concluded that this substance must be storage stable. O'Brien[27] found a thromboplastic factor in serum and, from a series of isolation experiments, concluded that it must originate from lysed platelets.

The Stypven coagulation time studies also demonstrate this platelet-like activity in the stored plasma. O'Brien[10] showed that the active platelet factor that caused plasma to clot when reacted with viper venom, was in the lipid fraction of the platelet extract. He believed it to be phosphatidyl-ethanolamine, but later work showed that the active principle is phosphatidyl-serine.

It is apparent from the studies reported here that this platelet thromboplastic factor is stable, and acts independently of the presence of viable or even of intact platelets.

Clot retraction. Clot retraction is generally believed to be a platelet function. The exact mechanism by which the platelets affect the fibrin strands to produce retraction is not well understood. One theory holds that the fibrin strands shorten when acted upon by an enzyme (retractozyme) that is released during platelet lysis. Another hypothesis states that clot retraction is the result of a direct physical action of the platelet processes on the fibrin mesh, and this action requires the presence of viable platelets.

The dissociation of the various platelet functions has been noted previously.[11,21] In contrast to the storage-stable thromboplastic factor, most authors agree that clot

retraction decreases greatly within 10 to 24 hours after phlebotomy. However, results reported here show that clot retraction remained excellent through 48 hours. After 72 hours, clot retraction decreased notably, even though good thromboplastic activity was retained by the same platelets. This loss of platelet function coincided with the appearance in the stained smears of the small, basophilic platelet forms previously described.

The life span of normal platelets appears to be about three days, as measured by various radiophosphorus technics.[28,29] Hirsch, Gardner, and Thomas[30] reported in vitro life span of platelets as being from 36 to 72 hours, as measured by glycolytic activity. The decrease in oxygen consumption is roughly correlated with a decrease in clot retraction. Stefanini and Dameshek[11] noted correlation between clot retraction and platelet survival in vivo. In all these studies, if the platelets were washed, concentrated, or otherwise were disrupted, there was a significant decrease in viability, which was roughly correlated to a decline in clot retraction.

The reports of Krevans and Jackson,[3] and Jackson, Krevans, and Conley,[4] state that most platelets remain viable only about four hours in ACD solution, and that after this time, if they are transfused they are rapidly removed from the circulation. However, in their studies,[3,4] the blood was taken into nonsiliconized equipment. Finkbiner and associates[15] support this observation, and state further that in surgical procedures requiring multiple transfusions, each alternate bottle of blood should be less than 24 hours old.

In contrast, Stefanini and Dameshek[11] noted that if platelets were stored in siliconized nonvacuum bottles, more than one half remained in the circulation at 12 hours. If the platelets were washed, or otherwise subjected to trauma, there was poor survival in vivo. Adelson, Rheingold, and Crosby,[28] utilizing radiophosphorus-tagged platelets, showed that excess trauma to the platelets led to a rapid decrease in the half-life in vivo. Hirsch, Gardner, and Thomas[30] noted that the oxygen consumption of platelet suspensions in ACD solution did not reach zero until after three days of storage. Mustard[20] stated that trauma to the platelets during phlebotomy greatly shortened their survival during storage in ACD solution. Of 62 patients, those in whom there was a decrease in circulating platelets had received blood that showed the greatest decline in platelet numbers during storage. In contrast, when such patients were transfused with blood that had been collected by gravity into siliconized bottles, no thrombocytopenia was noted.

From the study here reported, as well as the other evidence cited, it is apparent that blood carefully drawn by gravity, through nonwettable donor sets into siliconized, glass containers, has the majority of the platelets preserved, and that these platelets remain viable 48 hours after phlebotomy. Further, clot retraction appears for the most part to correspond to viability of platelets in vitro, under the experimental conditions. That is, clot retraction measured on platelet-rich plasma roughly corresponds to platelet viability.

The question of in vivo survival of stored platelets, however, remains controversial. Independent studies by several authors have demonstrated that in vivo survival ranges from almost immediate disappearance to survival for as long as 24 hours. A further source of controversy is whether or not the transfused platelets, even if they survive, are able to function normally in the clotting mechanism. Many of the results described in the literature and reported at recent meetings seem to hinge on the conditions under which the platelet infusions are carried out. This has led to some disagreement in this area. Certainly the platelet survival in thrombocytopenic patients is greatly shortened, especially if they have undergone previous blood transfusions.

In a series of patients at the Cleveland Clinic Hospital who received more than 15,000 ml. of blood in an exceedingly short period of time at operation, those who received one unit of "fresh" blood from a siliconized nonvacuum bottle (0 to 48 hours old) for every three to four units of stored blood, demonstrated no clinical evidence of hypocoagulability, and clotting times (both Lee-White and ground glass) were within normal limits at the end of the procedure. Unfortunately, because of the emergency situation pertaining at surgery, no other detailed studies were carried out in these patients. When such a system was not utilized, similar patients almost invariably showed the typical hemorrhagic diathesis, which was not corrected by infusion of bottled blood, but only by direct blood transfusion utilizing the siliconized syringe technic.

It is, therefore, most important that spacing of fresh and older stored blood be scheduled for any procedure in which massive exsanguinating hemorrhage might be anticipated. If the hemorrhagic syndrome does develop, immediate direct blood transfusion is indicated.

II. *Other clotting factors. AHG (antihemophilic-globulin).* These studies showed a rapid decline in AHG activity during the first 24 hours of storage. Pitney and Dacie[31] stated that this loss may be as much as 50 per cent in 24 hours; a recent report[15] stated that 50 per cent loss does not occur until blood has been stored for one week. Mustard[32] showed that loss of AHG activity is proportional to platelet loss during collection, and related this loss to activation of the clotting mechanism.

There is a definite decline in AHG during storage. From this study it would seem difficult to evaluate the exact amount of AHG lost, or the rate of decline in its activity, because of the concomitant increase in plasma thromboplastic activity that has been herein demonstrated. As noted before, this seems to be secondary to platelet lysis. It is probable that through 72 hours of storage, the loss of AHG activity is not enough to become a factor in the hemorrhagic diathesis that may occur during massive blood transfusion therapy.

Prothrombin. Fahey, Ware, and Seegers,[33] and Bell,[34] showed that prothrombin is storage stable in ACD solution. Finkbiner and associates[15] state that the average prothrombin concentration is 72 per cent of normal in blood stored from 11 to

18 days. This study showed only minimal loss of prothrombin activity during 14 days of storage at 4 C. Hypoprothrombinemia is not a factor in the hemorrhagic syndrome secondary to transfusion.

Factor V. Factor V was extremely storage labile, producing a prolonged prothrombin time in more than one half the samples tested after 12 hours of storage. This lability seemed independent of the platelet count, platelet activity, or plasma thromboplastic activity. Mustard[32] related the rate of factor V loss directly to the numbers of platelets lost during phlebotomy. Fahey, Ware, and Seegers[33] found that the addition of platelet extract or decalcified human plasma decreased the storage stability of factor V, and that factor V is more storage stable in platelet-free than in platelet-rich plasma. Finkbiner and associates[15] noted only a 10 per cent decrease in the concentration of factor V after 12 hours of storage, with a steady decline thereafter. He also noted that in patients with hepatic disease (who already have a factor V deficiency) transfusion of stored blood decreased the factor V value of these patients even further. It is probable that in patients with normal hepatic function, the use of blood stored as long as 96 hours would not cause any serious factor V deficiency.

Recalcified clotting time. Despite the deficiencies of individual factors in the clotting mechanism caused by storage, the recalcified clotting time in many instances remained within normal limits. During the first 72 hours it was rarely prolonged more than 10 seconds. Mustard[23] also commented on this phenomenon; but whether or not it is due to the insensitivity of the test, to the presence of platelet or of plasma thromboplastic factor in the stored blood, or to the formation of highly active intermediate products during storage, is in the realm of speculation at this time. However, it seems that the test for recalcified clotting time is not an adequate guide to the status of the coagulation system of stored blood in vitro.

Conclusions

The clotting mechanism of blood carefully drawn through siliconized equipment into a nonvacuum siliconized ACD solution bottle, and stored at 4 C., remains essentially intact during a period of 48 hours, despite a decrease in some coagulation factors, as tested in vitro. This is especially true of the platelets, which appear to be the critical element in the production of hemorrhagic diathesis during massive blood transfusion therapy. This is in contrast to blood drawn into nonsiliconized vacuum bottles, which showed a great decrease in platelets immediately postphlebotomy, and a rapid fall in platelet numbers thereafter.

This increased preservation of the clotting factors is probably due to the prevention of platelet lysis and the activation of Stuart factor against wettable surfaces. This, in turn, means that the activation of the clotting mechanism is retarded, and less thrombin is produced. Since thrombin, in turn, increases platelet lysis, less thrombin production means less platelet destruction. When coagulation

is retarded, such substances as antihemophilic-globulin (AHG) and factor V are spared.

Blood drawn and processed by the technic described in this paper may be considered as fresh blood through 48 hours of storage at 4 C. When an emergency need arises for massive transfusion therapy (4000 ml. or more of whole blood) it is recommended that one unit of such blood be utilized for every three units that have been stored more than 48 hours, or which have been drawn into vacuum nonsiliconized containers. If such a technic is followed, hemorrhagic diathesis will generally be avoided.

Patients who received more than 15,000 ml. of blood in an extremely short time, have been transfused in this fashion here at the Cleveland Clinic. No hemorrhagic phenomena due to hypocoagulability have been observed, and the clotting time at the end of the procedures has been normal in these patients.

This system of transfusion for emergency procedures has the advantage of giving the blood bank personnel time to locate and carefully to type and to cross-match donors in an orderly fashion, thus insuring a high quality of work at a time of extreme pressure.

The procedures and equipment recommended in this report can be easily adapted to most blood banks already using glass vacuum containers, with relatively little increase in unit cost. It is especially adaptable to the medium-sized or smaller blood banks where blood from volunteer repay donors and professional donors is collected and processed.

Acknowledgment

The author gratefully acknowledges the advice and aid given him in these studies by Dr. George C. Hoffman of the Department of Clinical Pathology, the Cleveland Clinic.

References

1. Stefanini, M., and Chatterjea, J. B.: Studies on platelets; thrombocytopenic factor in normal human blood, plasma, or serum. Proc. Soc. Exper. Biol. & Med. **79:** 623-629, 1952.

2. Stefanini, M.; Mednicoff, I. B.; Salomon, L., and Campbell, E. W.: "Thrombocytopenia of replacement transfusion": cause of surgical bleeding. Clin. Res. Proc. **2:** 61, 1954.

3. Krevans, J. R., and Jackson, D. P.: Hemorrhagic disorder following massive whole blood transfusions. J. A. M. A. **159:** 171-177, 1955.

4. Jackson, D. P.; Krevans, J. R., and Conley, C. L.: Mechanism of thrombocytopenia that follows multiple whole blood transfusions. Tr. A. Am. Physicians **69:** 155-162, 1956.

5. Wintrobe, M. M.: Clinical Hematology. 4th ed. Philadelphia: Lea & Febiger, 1956, 1184 pp.; pp. 267-268.

6. Wilson, S. J.; Eisemann, G., and Chance, J. H.: Plasma "thrombocytopenic factor" as measured by direct and indirect platelet methods. Proc. Soc. Exper. Biol. & Med. **81:** 317-319, 1952.

7. Biggs, R., and Douglas, A. S.: Thromboplastin generation test. J. Clin. Path. **6:** 23-29, 1953.

8. Quick, A. J.: On quantitative estimation of prothrombin. Am. J. Clin. Path. **15:** 560-566, 1945.

9. Jenkins, J. S.: Thromboplastic activity of Russell's viper venom and its relationship to factor VII. J. Clin. Path. **7:** 287-289, 1954.

10. O'Brien, J. R.: Similarity of action of phosphatidyl-ethanolamine and platelets in blood coagulation. J. Clin. Path. **9:** 47-51, 1956.

11. Stefanini, M., and Dameshek, W.: Collection, preservation and transfusion of platelets with special reference to factors affecting "survival rate" and clinical effectiveness of transfused platelets. New England J. Med. **248:** 797-802, 1953.

12. Mustard, J. F., and Walker, C. B.: Influence of blood collecting techniques on platelet numbers during blood storage. Brit. J. Haemat. **3:** 50-54, 1957.

13. Stefanini, M.: Mechanism of blood coagulation in normal and pathologic conditions. Am. J. Med. **14:** 64-86, 1953.

14. Lewisohn, R.: Blood transfusion by citrate method. Surg. Gynec. & Obst. **21:** 37-47, 1915.

15. Finkbiner, R. B.; McGovern, J. J.; Goldstein, R., and Bunker, J. P.: Coagulation defects in liver disease and response to transfusion during surgery. Am. J. Med. **26:** 199-213, 1959.

16. Pepper, H., and Lindsay, S.: Responses of platelets, eosinophils and total leucocytes during and following surgical procedures. Surg. Gynec. & Obst. **110:** 319-326, 1960.

17. Desforges, J. F.; Bigelow, F. S., and Chalmers, T. C.: Effects of massive gastrointestinal hemorrhage on hemostasis. I. Blood platelets. J. Lab. & Clin. Med. **43:** 501-510, 1954.

18. Warren, R.; Lauridsen, J., and Belko, J. S.: Alterations in numbers of circulating platelets following surgical operation and administration of adrenocorticotropic hormone. Circulation **7:** 481-486, 1953.

19. Mustard, J. F.: Changes in platelet levels of non-transfused patients following surgical operations. Acta haemat. **17:** 257-262, 1957.

20. Mustard J. F.: Effect of stored blood transfusions on platelet levels in patients undergoing surgical procedures. Acta haemat. **18:** 80-97, 1957.

21. Tullis, J. L.: Preservation of platelets. Am. J. M. Sc. **226:** 191-202, 1953.

22. Zucker, M. B., and Borrelli, J.: Reversible alterations in platelet morphology produced by anticoagulants and by cold. Blood **9:** 602-608, 1954.

23. Mustard, J. F.: Platelets in stored blood. Brit. J. Haemat. **2:** 17-24, 1956.

24. Haydon, G. B.: Electron microscopic observations of blood platelets and fibrin formation. A. M. A. Arch. Path. **64:** 393-397, 1957.

25. Minor, A. H., and Burnett, L.: Method for separating and concentrating platelets from normal human blood. Blood **7:** 693-699, 1952.

26. Stefanini, M.; Dameshek, W., and Adelson, E.: Platelets III. Shortened "platelet survival time" and development of platelet agglutinins following multiple platelet transfusions. Proc. Soc. Exper. Biol. & Med. **80:** 230-235, 1952.

27. O'Brien, J. R.: Platelet-like activity of serum. Rev. hémat. **10:** 347-349, 1955; Brit. J. Haemat. **1:** 223-228, 1955.

28. Adelson, E.; Rheingold, J. J., and Crosby, W. H.: Studies of platelet survival by tagging in vivo with P^{32}. J. Lab. & Clin. Med. **50:** 570-576, 1957.

29. Leeksma, C. H. W., and Cohen, J. A.: Determination of the life of human blood platelets using labelled diisopropylfluorophosphonate. Nature, London, **175:** 552-553, 1955.

30. Hirsch, E. O.; Gardner, F. H., and Thomas, E. D.: Isolation and concentration of human blood platelets: their properties in vitro and in vivo. J. Clin. Invest. **31:** 638-639, 1952.

31. Pitney, W. R., and Dacie, J. V.: Haemophilia and allied disorders of blood coagulation. Brit. M. Bull. **11:** 11-16, 1955.

32. Mustard, J. F.: Study of changes in platelets, antihaemophilic globulin, factor V and factor VII during blood collection and storage by different techniques. Brit. J. Haemat. **3:** 202-214, 1957.

33. Fahey, J. L.; Ware, A. G., and Seegers, W. H.: Stability of prothrombin and Ac-globulin in stored human plasma as influenced by conditions of storage. Am. J. Physiol. **154:** 122-133, 1948.

34. Bell, W. N.: Clinical use of coagulogram. M. Clin. North America **37:** 1843-1853, 1953.

A GUIDE FOR DIETARY REDUCTION OF SERUM CHOLESTEROL LEVEL IN PATIENTS WITH HYPERLIPEMIA

HELEN B. BROWN, Ph.D.
Division of Research

THE dilemma of the practitioner in choosing a satisfactory method of controlling blood lipids is illustrated by Dr. Philip L. White[1] in his introduction to the *Symposium on Significance of Lowered Cholesterol Levels:* "Pressure from the patients and their families, pressure from the promoters of certain drugs, and his own determination to apply the most beneficial measures to these patients have left the physician in a frustrating position." One member of the panel suggests that patients and their families should rely on the "informed individual physician," but, through no fault of his own, the individual physician is often not informed. Out of the welter of suggestions for reducing blood lipids, he often finds the dietary approach the most practical.

It is true we cannot speak conclusively about the dietary factors influencing serum lipids, the optimum proportion of nutrients, the chemical characteristics of fats causing serum lipid changes, or the long-term benefits of treatment. However, we are currently justified in using the knowledge obtained from well-controlled dietary experiments to reduce the patient's blood cholesterol.

Let us follow a hypothetic patient with evidence of atherosclerotic disease. We first make sure he is a good candidate for dietary treatment. We rule out conditions that temporarily alter serum cholesterol level. Weight loss, diarrhea, fever, and medications such as desiccated thyroid, estrogens, nicotinic acid, or sitosterol reduce serum cholesterol levels temporarily; whereas rapid weight gain, cortisone, testosterone, or corticotropin (ACTH) raise it. He is not on a self-imposed diet, another possible factor influencing serum lipid levels. We find his serum cholesterol concentration is in the neighborhood of 375 mg. per 100 ml. on two separate occasions. He eats in restaurants only occasionally; he has a concerned and co-operative wife; he takes his own lunch to work, or eats lunch regularly at one restaurant where his special needs will be met. This patient is a good candidate for dietary instruction and treatment.

It is useless and even harmful, to the patient's morale, to prescribe a diet that he cannot follow within his established daily routine. The traveling man who eats most of his meals in hotels and restaurants cannot adhere to food restrictions, however co-operative he may be. Others may by psychologically unable to accept new food patterns, or their living situations may be such that the suggested changes would do more harm than good.

What food pattern should be prescribed? The physician has two choices for this patient, either of which changes the accustomed fat yet supplies all essential nutrients.[2] The *low-fat* diet limits *all fats* to 13 per cent of total calories. For

example, in a 2000-calorie low-fat diet, 15 gm. of animal fat is allowed, and 15 gm. from other sources, mainly margarine at the table and hydrogenated shortening in cooking. The other choice is the *vegetable-oil* diet in which total fat remains at the average American level of 40 per cent of the calories; vegetable oils are substituted for the usual animal and hydrogenated fats. The vegetable-oil food pattern supplying 2000 calories contains 15 gm. of animal fat as in the low-fat diet and, in addition, 75 gm. of fat from vegetable oils.

The choice between the low-fat and the vegetable-oil food patterns usually depends upon which is more adaptable to the patient and his living habits. In general, the serum cholesterol levels are lower and more stable with the vegetable-oil food pattern. For example, on the low-fat diet, occasional fatty foods cause a considerable rise in serum cholesterol level, but a five-day vacation from vegetable oil brings about only a minor change. However, as indicated below, a few patients have a lower cholesterol level on the low-fat diet than on the vegetable-oil diet. When weight reduction is necessary, the low-fat diet may be prescribed at first, and when appropriate weight is attained, the patient then may change to the vegetable-oil diet. Most patients, after a time, find the low-fat regimen rigorous. The vegetable-oil food pattern is closer to the ordinary American bill of fare, affording variety, palatability, and satisfaction.

In both food patterns, animal fat is eliminated by avoiding butterfat, by using egg yolks only occasionally in simple desserts, by cooking with the minimum of fat, by selecting low-fat foods, and by choosing lean meat, fish, and poultry. Lean meat is easily recognized by its lack of marbling. Visible fat is trimmed before cooking, and the liquid fat is drained off during cooking. No particular attempt is made to reduce the cholesterol content of the foods.

The vegetable-oil food pattern requires not only reduction of all animal fat, as in the low-fat diet, but, in addition, substitution of vegetable oil for the usual hard fats, five to seven times as much oil as animal fat. In practical terms, the amount of vegetable oil varies from 4 to 7 tablespoonfuls a day, depending on the caloric requirement. A useful rule of thumb is to prescribe 4 or 5 tablespoonfuls for a sedentary adult, 6 or 7 tablespoonfuls for large and active persons. Cottonseed, corn, peanut, soybean oils are all suitable. The oil should not be taken as a medicine, but can be combined in foods in many ways: as a nonhydrogenated spread, salad dressing, seasoning for vegetables and meats, in sauces, and as shortening in breads, cakes, cookies, and pies.

For reduction of serum cholesterol, it is most important to limit all kinds of fat, *including vegetable oils,* in the low-fat diet. In the vegetable-oil diet, there must be a considerable *excess of vegetable oil.* These relationships[3] must be held firmly in mind. What happens when 30 gm. of an unsaturated oil preparation is taken in addition to freely chosen foods? Animal and hydrogenated fats are still in excess of oil. When 30 gm. of an oil preparation is added to the low-fat diet, a common practice at the present time, the resulting diet will have 60 gm. of fat, so it is no

longer low in fat, yet it is not high enough in vegetable oil to be effective in reducing serum cholesterol levels.

What sort of serum cholesterol change can be expected in this patient with dietary treatment over a year's time? The answer depends upon his type of hyperlipemia.[4] There are three different types. The first is the commonly accepted hyperlipemia that we prefer to call *hyperglyceridemia,* a more exact, descriptive term; the second is the commonly recognized *hypercholesteremia;* the third is a recently described[4] category we have called *mixed hyperlipemia.* These serum lipid abnormalities are characterized by variations in relationship between the serum cholesterol, triglycerides, and phospholipids. Serum cholesterol concentration in all three types is elevated above normal. In hyperglyceridemia, the triglyceride fraction is much higher than cholesterol and phospholipid fractions. In hypercholesteremia, cholesterol is high out of all proportion to the triglyceride and phospholipid fractions. In mixed hyperlipemia, cholesterol, triglyceride, and phospholipid all are high, but in normal proportions to each other.

How can a physician tell what serum lipid abnormality the patient has without a great many laboratory tests? Determinations of cholesterol and lipid phosphorus with visual examination of a fasting sample are usually sufficient to distinguish among the three types of hyperlipemia. In hyperglyceridemia, serum is usually milky, the total cholesterol to phospholipid ratio is normal, less than 1.0, while in hypercholesteremia, serum is clear, and the total cholesterol to phospholipid ratio is high, greater than 1.2. In mixed hyperlipemia, serum is clear or somewhat opalescent, and the proportion of cholesterol to phospholipid is 1.0 or less.

The three types of hyperlipemia differ in ways other than in the lipid pattern. In hyperglyceridemia, the serum cholesterol level is changeable; cholesterol concentration varies as much as 100 to 200 mg. per 100 ml. from one time to another for no apparent reason. In hypercholesteremia, serum cholesterol is stable, even when there is rapid weight gain. One patient who gained 20 pounds in less than two months had no more than 10 mg. of difference in cholesterol levels. In mixed hyperlipemia, serum cholesterol levels vary normally, ± 25 mg. per 100 ml. in a year.

The three types of hyperlipemia differ in their responses to dietary treatment. In patients with hyperglyceridemia who follow the low-fat diet, serum cholesterol levels become nearly normal, but remain highly variable. Cholesterol usually is lowered and more stable on the vegetable-oil food pattern. Vegetable-oil is the preferable food pattern for patients of this group, except for a few who have lower serum cholesterol levels on the low-fat diet. In hypercholesteremia, serum cholesterol may be reduced by either diet, though in many patients it will not fall below 350 mg. per 100 ml. In other patients, serum cholesterol drops to 300 mg. or less per 100 ml., but may revert to higher levels after from three to six months of dietary treatment. Patients with hypercholesteremia require treatment other than diet to attain a normal serum cholesterol concentration; as yet, no

such treatment has been found. For some patients, the vegetable-oil diet elicits a lower serum cholesterol level than does the low-fat diet. In patients with mixed hyperlipemia, serum cholesterol levels decrease readily with the use of either food pattern, and remain normal for as long as they remain on the diet. Some of them, in our experience, have maintained normal serum cholesterol levels for more than two years.

Differences of opinion will arise as to the value of dietary treatment of elevated serum cholesterol levels, unless the types of lipid abnormality are taken into consideration and an adequate food pattern is employed. When treating this hypothetic patient for a reduction of a high serum cholesterol level by changing his food pattern, two questions must be asked: What type of hyperlipemia does he have? What food pattern will be most effective for him? Failure to ask these questions—and to find the right answers—is often responsible for the physician's discouragement with dietary management of hyperlipemia.

References

1. White, P. L.: Introduction, p. 2198, *to* Symposium on significance of lowered cholesterol levels. J. A. M. A. **170:** 2198-2203, 1959.

2. Brown, H. B., and Page, I. H.: Lowering blood lipid levels by changing food patterns. J. A. M. A. **168:** 1989-1995, 1958.

3. Brown, H. B.: Diet and hypercholesteremia; comment. Cleveland Clin. Quart. **25:** 191-196, 1958.

4. Brown, H. B., and Page, I. H.: Variable responses of hyperlipemic patients to altered food patterns. J. A. M. A. **173:** 248-252, 1960.

THE CLINICAL APPLICATION OF A MODIFIED AZO-DYE TECHNIC FOR THE DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY IN NEUTROPHILS

GEORGE C. HOFFMAN, M.A., M.B., B.CHIR., and
VIRGINIA J. LUCICH, M.T. (A.S.C.P.)
Department of Clinical Pathology

THE phosphatases are a group of enzymes that release orthophosphoric acid from many phosphate esters and, to a lesser extent, participate in the reverse reaction. These enzymes differ in their optimal pH and, on this basis, they may be classified as either acid or alkaline phosphatases. In 1939, Gomori[1] and Takamatsu[2] independently described a method for the cytochemical demonstration of alkaline phosphatase. Since that time the distribution of alkaline phosphatase in the cells of the hematopoietic system has been the subject of much study. In 1944, Menten, Junge, and Green[3] described an azo-dye method for the demonstration of the enzyme. The basic reactions in the technic are the release of naphthol from an alpha-naphthyl phosphate substrate, and its combination with a diazotized amine to form an azo dye. This dye forms a colored precipitate at the site of action of the alkaline phosphatase within the cells.

Reports[4-8] differ both as to the types of cell that contain alkaline phosphatase and as to the distribution of the enzyme within the individual cells. Nevertheless, most authors agree that it is the alteration in enzyme content of the segmented neutrophils which offers the most useful diagnostic information. In chronic granulocytic leukemia the enzyme activity of the segmented neutrophils has been shown[4-14] to be greatly reduced or absent, whereas in other forms of granulocytosis this activity is frequently increased. Polycythemia vera,[15] myelofibrosis,[13,14] Hodgkin's disease[15] and pregnancy[16] are also associated with increased neutrophil alkaline phosphatase.

The purpose of this paper is to present a modified azo-dye technic for the cytochemical measurement of alkaline phosphatase, and to discuss its use as a diagnostic aid.

Method

A modification of the azo-dye methods described by Kaplow[7] and by Hayhoe and Quaglino[8] was used for the semiquantitative measurement of alkaline phosphatase in segmented neutrophils.

Films of freshly drawn blood are spread on chemically clean glass slides; the enzyme activity of the neutrophils falls rapidly if the blood is drawn into an anticoagulant solution. The films are fixed for 90 seconds in a mixture of 10 per cent formalin in absolute alcohol at 5 C. A fixation time of 90 seconds was found to lead to a more distinct staining reaction than a fixation time of 30 seconds.

Fixed blood films can be stored overnight prior to staining, however unfixed blood films are unsatisfactory if stored for this length of time. The fixative must be stored at —20 C. and should be freshly prepared each week. After fixation, the blood films are rinsed in tap water and stained for 10 minutes at room temperature with the following mixture:

Sodium alpha-naphthyl phosphate.....35 mg.
Brentamine Fast Garnet*.....35 mg.
0.05M propanediol buffer.....35 ml.

This mixture must be prepared immediately before use and should be filtered directly onto the slide. After rinsing in distilled water the blood film is counterstained with Mayer's hematoxylin for 10 minutes. The estimation of alkaline phosphatase activity was found to be simpler when Mayer's hematoxylin rather than methyl green was used as a nuclear counterstain. The stained film is mounted in Kaiser's glycerine jelly, a mounting medium that considerably slows the rate at which the azo dye fades. The color can be preserved for even longer periods if the stained film is fixed in absolute alcohol prior to mounting.

When alkaline phosphatase is present in a cell *(Fig. 1)* it is represented by a red-brown precipitate in the cytoplasm. The amount of this precipitate varies

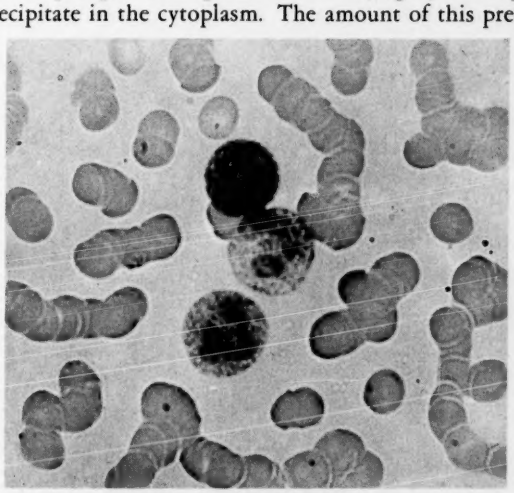

Fig. 1. A photomicrograph of three segmented neutrophils showing alkaline phosphatase activity represented by a (red-brown) precipitate of varying density in the cytoplasm. The upper neutrophil is graded 4+, the central neutrophil, 2+, and the lower neutrophil, 3+. Magnification x 1000.

from a faint diffuse brown coloration to a dense aggregation of red-brown granules. Using the method of scoring described by Kaplow,[7] this variation in staining can be used roughly to quantitate the amount of enzyme that is present in the cell. One hundred consecutive segmented neutrophils are individually scored in a

**Imperial Chemical Industries Ltd., England.*

range from 0 to 4+. These individual scores are totaled and the resulting figure is the score for the blood being studied. Thus the range of possible scores is 0 to 400.

Results

The various disorders and the number of patients studied are listed in *Table 1.* The individual alkaline phosphatase scores obtained in the larger groups are charted in *Figure 2.*

Table 1.—*The number of patients* studied and their respective diagnoses*

Diagnosis	Patients studied, number
Normal	30
Chronic granulocytic leukemia	13
Other chronic leukemias	6
Acute leukemias	7
Benign leukocytosis	15
Polycythemia vera	30
Anoxemic erythrocytosis	10
Myelofibrosis and allied disorders	6
Hodgkin's disease	20
Other lymphomas	8
Disseminated lupus erythematosus	10
Primary refractory anemia	4
Pernicious anemia in relapse	2
Hyperthyroidism	3
Hypothyroidism	3
Pregnancy	2
Granulocytopenia (drug-induced), acquired hemolytic anemia, thrombotic thrombopenic purpura, sickle-cell anemia, infectious mononucleosis	1 each

*Many of these patients were under the care of Dr. John D. Battle, Jr., and Dr. James S. Hewlett, of the Department of Hematology.

Alkaline phosphatase activity was seen in neutrophilic myelocytes, metamyelocytes, band forms, and, more commonly, in segmented neutrophils. The only other cells to show activity were the reticuloendothelial cells of the bone marrow. Alkaline phosphatase was found to be restricted to the cytoplasm and was never seen in the nucleus. Since the counterstained nucleus was visible even in cells

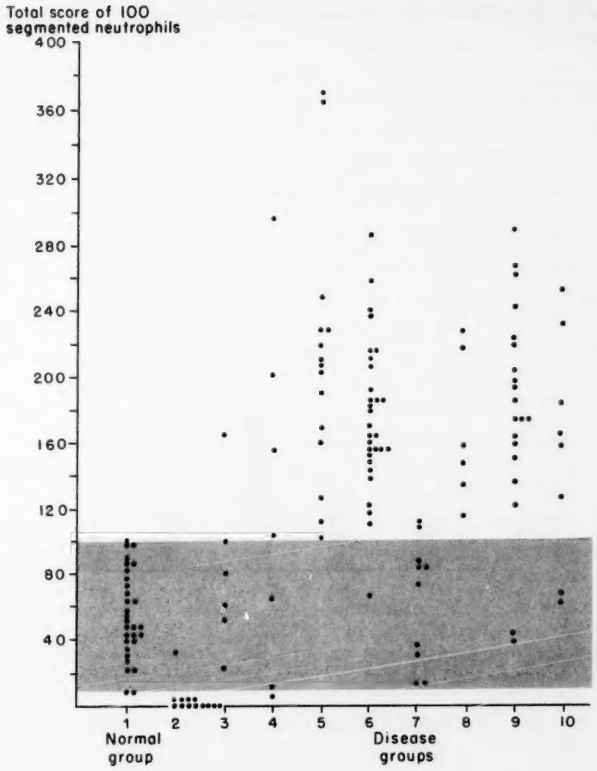

Fig. 2. A chart showing the individual alkaline phosphatase scores obtained in a group of normal persons and in patients with various diseases. The normal range is shaded. Key: 1 = normal; 2 = chronic granulocytic leukemia; 3 = other chronic leukemias; 4 = acute leukemias; 5 = non-leukemic leukocytosis; 6 = polycythemia vera; 7 = anoxemic erythrocytosis; 8 = other myeloproliferative disorders; 9 = Hodgkin's disease; 10 = other lymphomas.

graded 4+, the identity of the individual cells was seldom in doubt. The only exception was the basophil, which was not clearly recognizable. Eosinophils, which were recognized by their nuclear structure, showed no alkaline phosphatase activity.

Normal persons. Peripheral blood films from 30 normal persons were studied, and the alkaline phosphatase scores ranged from 8 to 100. No cells with a 4+ rating were seen in this group, and less than 1 per cent of the neutrophils was rated 3+. The greatest number of cells rated 3+ in any one normal blood film

was 3 per cent. This was in distinct contrast to that found in diseases associated with a high score (*Fig. 3*).

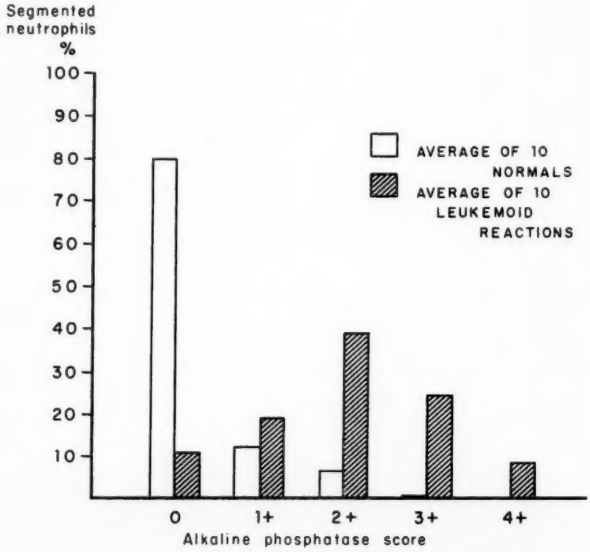

Fig. 3. A comparison of the distribution of neutrophil alkaline phosphatase scores (0 to 4+) in a group of 10 normal persons and a group of 10 patients with leukemoid reactions.

During the study, pharyngitis developed in two of the normal subjects and, although the leukocyte counts remained within normal limits, the alkaline phosphatase scores rose to 130 and 190. When the symptoms subsided the scores returned to 83 and 36, respectively.

Chronic granulocytic leukemia. Eight of thirteen patients with chronic granulocytic leukemia had scores of zero, and 12 had scores of 3 or less. Four of the 12 patients, untreated at the time of study, had leukocyte counts ranging from 89,500 to 373,000 per cubic millimeter. The remaining eight patients had been under treatment for from four months to four years and had leukocyte counts ranging from 4,800 to 18,900 per cubic millimeter. The one patient with a normal score of 33 had been treated with busulfan for six months and had a normal leukocyte count.

Other leukemias. The five patients with chronic lymphocytic leukemia showed a wide range of scores from 22 to 163. There was no relationship between the alkaline phosphatase score and the total leukocyte count, the neutrophil count, or the stage of the disease. One patient with chronic monocytic leukemia had a score of 60.

Seven patients with acute leukemia also showed a wide range of scores. Two patients with acute granulocytic leukemia had scores of 6 and 154; four patients with acute lymphocytic leukemia had scores of 11, 64, 102, and 201; one patient with acute monocytic leukemia had a score of 295.

Nonleukemic leukocytosis. Nonleukemic leukocytosis almost invariably was associated with alkaline phosphatase scores above the normal range. Nine patients with leukocytosis due to infection had scores ranging from 111 to 372. In this group there was a rough correlation between the neutrophil count and the alkaline phosphatase score. Three patients with carcinomatosis and leukocytosis had scores of 169, 218, and 367. Two other patients with carcinomatosis and normal leukocyte counts had scores of 140 and 216; these two scores are not included in *Figure 2.* All five patients with carcinomatosis showed some evidence of infection.

Three patients with leukocyte counts of 16,000, 20,000, and 31,000 per cubic millimeter showed no evidence of any underlying disease and had high leukocyte counts for at least one year. Their scores were 160, 249, and 103, respectively.

Polycythemia vera and anoxemic erythrocytosis. Thirty cases of polycythemia vera were studied and with one exception (score of 67) the scores were more than 110. Twenty of these patients had leukocyte counts of less than 10,000 per cubic millimeter, and their scores ranged from 67 to 258 with an average of 170. Ten patients had leukocyte counts of more than 10,000 per cubic millimeter, and their scores ranged from 143 to 288 with an average of 187. These differences are of little significance. Similarly, a comparison of the scores obtained in treated and in untreated patients showed no significant difference. In most instances the treated patients had received radioactive phosphorus therapy for months or years, and had normal peripheral blood counts. The score of 67 was obtained in a patient who has required no treatment for four years.

In contrast to the findings in patients with polycythemia vera, 10 patients with anoxemic erythrocytosis had scores ranging from 11 to 112 with an average of 65.

Other myeloproliferative disorders. The group of six patients with myeloproliferative disorders, other than polycythemia vera or chronic granulocytic leukemia, comprised: three patients with myelofibrosis and myeloid metaplasia; one patient with myelofibrosis and myeloid metaplasia secondary to a metastasizing carcinoma of the breast; one patient with myelofibrosis, myeloid metaplasia, and mild polycythemia vera; and one patient with megakaryocytic myelosis. All six patients had alkaline phosphatase scores above the normal range, the lowest being 117 and the highest 229.

Hodgkin's disease and other lymphomas. Of 20 patients with Hodgkin's disease, 18 showed an increased alkaline phosphatase activity. There was no correlation between the alkaline phosphatase score and the total leukocyte count. Thus the six patients who had total leukocyte counts of less than 3,000 per cubic millimeter had scores ranging from 124 to 291. No correlation was found between the

enzyme activity, the stage of the disease, or the response to treatment.

Seven patients with lymphosarcoma were studied and their scores ranged from 65 to 254, five being above the normal range. One patient with a follicular lymphoma had a score of 196.

Other diseases. Ten patients with disseminated lupus erythematosus had alkaline phosphatase scores ranging from 61 to 258. Many of these patients had one or more complications such as hemolytic anemia, circulating anticoagulant, or thrombocytopenia. Two patients with untreated pernicious anemia had low scores of 8 and 12; their total leukocyte counts were 3,900 and 4,400 per cubic millimeter. Four patients with primary refractory anemia had scores of 5, 16, 24, and 111. Three patients with hyperthyroidism had scores of 33, 44, and 224. Three patients with myxedema had scores of 29, 49, and 220. Two women in the second and third trimesters of pregnancy had scores of 271 and 288. Single instances of acquired hemolytic anemia, sickle-cell anemia, thrombotic thrombopenic purpura, and infectious mononucleosis had scores within the normal range. One patient with rheumatoid arthritis, in whom a drug-induced granulocytopenia developed, was studied on two occasions. When initially studied the total leukocyte count was 2,100 per cubic millimeter with 10 per cent neutrophils. At that time the alkaline phosphatase score was 224. Two weeks later, when the total leukocyte count had risen to 8,000 per cubic millimeter with 54 per cent neutrophils, the alkaline phosphatase score was 93.

Discussion

The semiquantitative estimation of the alkaline phosphatase content of mature neutrophils by histochemical means has become established as a useful tool in the differential diagnosis of various hematologic disorders and certain other diseases. At present, the most useful application of this test appears to be in the differential diagnosis of chronic granulocytic leukemia and leukemoid reactions. The absence or great reduction of alkaline phosphatase activity is characteristic of chronic granulocytic leukemia, while the scores obtained in leukemoid reactions are normal or more commonly elevated. Although Tanaka, Valentine, and Fredricks[17] have shown that diseases other than chronic granulocytic leukemia may be associated with low enzyme activity, we have not found scores of 3 or less in any disease other than chronic granulocytic leukemia. The scores in the lower limits of the normal range occurred either in normal persons or in patients having diseases that are unlikely to be confused with chronic granulocytic leukemia. With one possible exception we have observed no return of alkaline phosphatase activity during a remission of chronic granulocytic leukemia. However, a return of enzyme activity has been reported by some authors.[8,13]

Myeloproliferative disorders, other than chronic granulocytic leukemia, such as myelofibrosis with myeloid metaplasia, megakaryocytic myelosis, and polycythemia vera are associated with high alkaline phosphatase scores. The high scores found in polycythemia vera often help to distinguish this form of erythro-

cytosis from the anoxemic type. A return to normal values following the successful therapy of polycythemia vera has been reported.[12] However, even in those patients who have been under excellent control for several years we have found only one normal score.

In contrast to the absent or reduced alkaline phosphatase activity found in chronic granulocytic leukemia, neutrophil leukocytosis due to any other cause is almost invariably associated with increased activity. Even a mild infection, unassociated with a significant leukocytosis, such as occurred in two of the group of normal persons, may be associated with a high score. The occurrence of increased neutrophil alkaline phosphatase under many nonspecific circumstances thus makes the interpretation of scores above the normal range difficult.

The increased alkaline phosphatase activity found in the various types of lymphoma may be of some diagnostic value. Hayhoe and Quaglino[8] found high scores in patients with Hodgkin's disease, and normal scores in patients with other types of lymphoma. We have been unable to make this distinction for, although we obtained consistently high scores in patients with Hodgkin's disease, we also obtained high scores in five of seven patients with other types of lymphoma.

No other useful diagnostic application for this test was found among the other conditions we have studied, with the possible exception of pregnancy. Quigley, Dawson, Bong, and Custer[16] have recently reported the interesting observation of high alkaline phosphatase scores at all stages of pregnancy, and they discuss the possibility of applying this finding to the diagnosis of early pregnancy. The only two pregnant women we have studied were in the second and third trimesters of pregnancy and had scores of 271 and 288.

Various theories have been propounded to explain the changes in the alkaline phosphatase content of the neutrophils that occur in various disorders. The different opinions that exist concerning the types of cell that contains alkaline phosphatase are no doubt in part due to differences in technic. These technics may not be measuring identical members of the alkaline phosphatase group of enzymes.[18] Trubowitz, Feldman, Benante, and Hunt[19] have reported high alkaline phosphatase scores following the administration of nitrogen mustard. They suggest that the high scores resulted from a cessation of production of young neutrophils, and the persistence in the peripheral blood of older neutrophils with high alkaline phosphatase activity. The same authors[20] noted higher scores in the segmented neutrophils of the peripheral blood than of the bone marrow. Both these findings indicate that alkaline phosphatase activity increases with the age of the neutrophil. Our findings in the patient with the transient drug-induced granulocytopenia may have a similar explanation. It can be postulated that the high score reflected an older population of neutrophils surviving from the period prior to bone marrow depression, and with the return of bone marrow activity and the production of younger neutrophils, the score returned to the normal range.

The leukocytosis that occurs in response to infection may, in part, result from

the release of relatively old neutrophils from sites of sequestration. However, the majority are younger cells arising directly from the bone marrow, and would not be expected to contain an increased amount of alkaline phosphatase if their age were the only factor. The high score that accompanies a leukocytosis due to infection must, therefore have some other explanation. Valentine and his associates[21] showed a direct correlation between adrenocortical activity and neutrophil alkaline phosphatase content. They suggest that this is a common denominator in various disorders, including pyogenic infections. The administration of 17-hydroxycorticosteroids will also increase the phosphatase activity of the neutrophils. We have studied a patient who received 200 gm. of prednisolone daily, and after three days of such treatment the enzyme score had risen from 79 to 143. Apart from its adrenocortical stimulating effect, a pyogenic infection may also increase the metabolic rate, and this might be reflected at the cellular level by an increase in alkaline phosphatase activity. To test this hypothesis, a small group of hyperthyroid and hypothyroid patients were studied. However, there was no significant correlation between metabolic rate and alkaline phosphatase activity of the neutrophils.

It is evident that more precise interpretation of the changes that occur in the alkaline phosphatase content of the neutrophils must await further knowledge concerning the physiologic factors affecting the enzyme at the cellular level. Nevertheless, useful clinical information can be gained from a study of the alkaline phosphatase content of the neutrophils.

Summary

A modified azo-dye technic for the cytochemical demonstration of the enzyme, alkaline phosphatase, in neutrophils is described.

This technic has been applied on a semiquantitative basis to peripheral blood films taken from normal persons and from patients with various disorders.

The absent or reduced alkaline phosphatase activity of the segmented neutrophils in chronic granulocytic leukemia, serves to distinguish this disease from other myeloproliferative disorders and from leukemoid reactions. Polycythemia vera with its increased alkaline phosphatase activity can be distinguished from other forms of erythrocytosis. The lymphomas are also associated with increased neutrophil alkaline phosphatase activity.

The findings in various other disorders are discussed with particular reference to the pitfalls that exist in interpreting increased alkaline phosphatase activity.

Acknowledgment

The authors wish to thank Miss Felicia A. Gbur, M. T. (A.S.C.P.), for her technical assistance.

References

1. Gomori, G.: Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exper. Biol. & Med. 42: 23-26, 1939.

2. Takamatsu, H. (Histochemical and biochemical studies of phosphatase. Tr. Jap. Path. Soc. **29:** 492, 1939.) Cited by Kaplow[7].

3. Menten, M. L.; Junge, J., and Green, M. H.: Coupling histochemical azo-dye test for alkaline phosphatase in kidney. J. Biol. Chem. **153:** 471-477, 1944.

4. Wachstein, M.: Alkaline phosphatase activity in normal and abnormal human blood and bone marrow cells. J. Lab. & Clin. Med. **31:** 1-17, 1946.

5. Rabinovitch, M., and Andreucci, D.: Histochemical study of "acid" and "alkaline" phosphatase distribution in normal human bone marrow smears. Blood **4:** 580-594, 1949.

6. Kerppola, W.: Observations on phosphatase content of blood and bone marrow cells in normal and pathological hemopoiesis. Blood **6:** 454-465, 1951.

7. Kaplow, L. S.: Histochemical procedure for localizing and evaluating leukocyte alkaline phosphatase activity in smears of blood and marrow. Blood **10:** 1023-1029, 1955.

8. Hayhoe, F. G., and Quaglino, D.: Cytochemical demonstration and measurement of leukocyte alkaline phosphatase activity in normal and pathological states by a modified azo-dye coupling technique. Brit. J. Haemat. **4:** 375-389, 1958.

9. Meislin, A. G.; Lee, S. L., and Wasserman, L. R.: Leukocyte alkaline phosphatase activity in hematopoietic disorders; correlative study by biochemical and cytochemical techniques. Cancer **12:** 760-766, 1959.

10. Brodell, H., and Swisher, S. N.: Studies of leukocyte alkaline phosphatase determined by clinically applicable histochemical method. Clin. Res. Proc. **2:** 58-59, 1954.

11. Kenny, J. J., and Moloney, W. C.: Leukocyte alkaline phosphatase; behavior during prolonged incubation and infection in normal and leukemic leukocytes. Blood **12:** 295-302, 1957.

12. Leonard, B. J.; Israëls, M. C., and Wilkinson, J. F.: Alkaline phosphatase in white cells in leukaemia and leukaemoid reactions. Lancet **1:** 289-292, 1958.

13. Mitus, W. J.; Bergna, L. J.; Mednicoff, I. B., and Dameshek, W.: Alkaline phosphatase of mature neutrophils in chronic forms of myeloproliferative syndrome. Am. J. Clin. Path. **30:** 285-294, 1958.

14. Koler, R. D.; Seaman, A. J.; Osgood, E. E., and Vanbellinghen, P.: Myeloproliferative diseases; diagnostic value of leukocyte alkaline phosphatase test. Am. J. Clin. Path. **30:** 295-301, 1958.

15. Mitus, W. J.; Mednicoff, I. B., and Dameshek, W.: Alkaline phosphatase of mature neutrophils in various polycythemias. New England J. Med. **260:** 1131-1133, 1959.

16. Quigley, H. J.; Dawson, E. A.; Bong, H. H., and Custer, R. P.: Activity of alkaline phosphatase in granular leukocytes during pregnancy and puerperium: preliminary report. Am. J. Clin. Path. **33:** 109-114, 1960.

17. Tanaka, K. R.; Valentine, W. N., and Fredricks, R. E.: Diseases or clinical conditions associated with low leukocyte alkaline phosphatase. New England J. Med. **262:** 912-918, 1960.

18. Burgos, M. H.; Deane, H. W., and Karnovsky, M. L.: Histochemical and chemical evidence for more than one alkaline phosphomonoesterase. J. Histochem. **3:** 103-121, 1955.

19. Trubowitz, S.; Feldman, D.; Benante, C., and Hunt, V. M.: Alkaline phosphatase content of human leukocytes following marrow suppression by nitrogen mustard. J. Lab. Clin. Med. **52:** 259-265, 1958.

20. Trubowitz, S.; Feldman, D.; Benante, C., and Hunt, V. M.: Alkaline phosphatase content of human polymorphonuclear leukocyte in blood and marrow. Am. J. Clin. Path. 31: 483-486, 1959.

21. Valentine, W. N., and others: Studies on leukocyte alkaline phosphatase activity: relation to "stress" and pituitary-adrenal activity. J. Lab. & Clin. Med. 44: 219-228, 1954.

THE USE OF OXYMORPHONE HYDROCHLORIDE* DURING ANESTHESIA FOR OPERATIONS ON THE HEAD AND NECK

A Preliminary Report

MERTON SEIGLEMAN, M.D.,† and CARL E. WASMUTH, M.D., LL.B.
Department of Anesthesiology

THIS paper is a brief report of our experience in the use of oxymorphone hydrochloride during anesthesia for surgical procedures involving the head and neck. Operations for cancer and procedures for cosmetic repair such as nasoplasty and septal reconstruction are included. No particular originality is claimed in respect to the technic described in this paper. We have found this technic to be safe for the patient and to be satisfactory to the surgeon in providing good operating conditions. It is believed that oxymorphone may well prove to be adaptable to operations involving not only the head and neck, but also other parts of the body.‡

The new synthetic, morphine-like alkaloid, 14-hydroxydihydromorphinone, has shown high analgesic potency on the basis of experimental and clinical investigation.[1] *Figure 1* shows the structural relationship between oxymorphone, morphine, and dihydromorphinone. Oxymorphone differs from morphine by the replacement of an alcoholic hydroxyl group by an oxygen atom at carbon 6 position, and attachment of a hydroxyl group at the fourteenth position, and from dihydromorphinone by a hydroxyl group in the fourteenth position.[2] The 7-8 bond is saturated in oxymorphone and unsaturated in morphine and dihydromorphinone.

The analgesic potency of oxymorphone in mice has been studied by Samuels, Stehlin, Dale, and Howe[3] using the hot-plate method of Eddy and Limbach injecting the drug subcutaneously. Oxymorphone was found to be 15 times as active as morphine and 2.5 times as active as dihydromorphinone.

In clinical studies Eddy and Lee[4] have shown that 2 mg. of oxymorphone is equivalent to 16 mg. of morphine sulfate, to 100 mg. of meperidine hydrochloride, or to 5 mg. dihydromorphinone. Respiratory depression appeared to be the most serious toxic symptom, but usually was minimal in patients who were not debilitated who received individual doses of no more than 5 mg. Nalorphine hydrochloride will rapidly counteract respiratory depression induced by oxymorphone.

The anesthesiologist must plan his technic so that the anesthesia is as light as feasible to allow prompt recovery even after long procedures. The technic must

**Numorphan hydrochloride, Endo Laboratories Inc.*

†Fellow in the Department of Anesthesiology.

‡Subsequent to the series reported in this paper, oxymorphone was employed with great efficiency in thoracic operations.

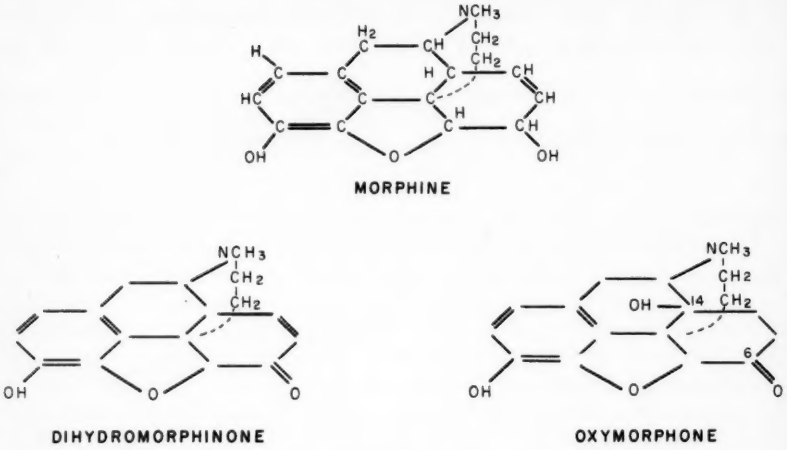

Fig. 1. Comparison of chemical structures of three anesthetic agents: morphine, dihydromorphinone, and oxymorphone.

not contribute to the deterioration of already existing pulmonary insufficiency. The aim of the anesthesiologist is to send the patient to the recovery room in possession of reflexes that will enable him to cope with blood and mucus in the trachea. This is of vital importance in patients who do not have a tracheotomy when an extensive surgical procedure and massive bandaging of the head and neck interfere with the airway.

In patients with neoplasm of the head and neck, the anesthesiologist preoperatively should acquaint himself with its locality and extent, and assess the amount of respiratory obstruction. He should study the roentgenograms and ascertain whether tracheal deviation is present.

The anesthetic agents selected should allow the patient to be asleep and free from pain. The respirations should be quiet, but the cough reflex and voluntary control of respiration must be retained. Since requirements for muscular relaxations are minimal, the use of relaxant drugs is limited.

Technic of Administration

Intravenous administration of anesthetics in a patient who has severe respiratory obstruction is extremely hazardous. In such a patient, a preliminary tracheotomy under local anesthesia is an essential precautionary measure. In suitable patients, the thiopental sodium—succinylcholine—oxygen sequence is employed prior to endotracheal intubation.

Premedication. In healthy adult patients, the usual premedication consisted of meperidine hydrochloride, 50 or 100 mg., and atropine sulfate, 0.6 mg. (1/100 gr.).

In extremely sick patients, only atropine sulfate was used. It is essential that atropine be used to protect the patient against the vagal stimuli resulting from surgical manipulation of the carotid sinus. In patients who have tachycardia, scopolamine, 0.5 mg. (1/150 gr.), may be substituted for atropine.

Anesthesia. Hypnosis was induced by 2 per cent solution of thiopental sodium, and initial muscular relaxation was provided by succinylcholine chloride, 50 mg., injected intravenously. The patient was given manual respiration with 100 per cent oxygen, and a cuffed endotracheal tube was inserted into the trachea. Maintenance of anesthesia was then provided by inhalation of a mixture of nitrous oxide and oxygen, of which 25 to 30 per cent was oxygen.

Adequate basal analgesia was provided by the intravenous injection of oxymorphone supplemented by inhalation of nitrous oxide and oxygen. The initial dose of oxymorphone was from 0.375 to 0.75 mg.; this amount was supplemented by additional doses of 0.375 mg. as required.

Results

In our series of 50 operations upon the head and neck, the outlined anesthetic technic produced good surgical operating conditions. The analgesia was profound and the patient could withstand the most powerful stimuli without showing signs of being disturbed. In spite of the severity of the surgical procedure and massive bandages, the patient maintained his airway and spontaneous breathing. In addition, he was tranquil and free of pain, the state characteristically induced by oxymorphone. At the conclusion of the operation, each patient was awake and in control of his vital protective reflexes; he was easily aroused, and, when requested, coughed.

Side effects. The side effects after intravenous injections of oxymorphone varied with the individual patient, and involved decrease in blood pressure and depressed respiration. In some patients there was an initial fall in systolic blood pressure of as much as 20 mm. of Hg. The decrease in blood pressure was never severe and in most patients was corrected spontaneously; an intravenous injection of a 5-mg. dose of methoxymine hydrochloride* restored normal blood pressure in the others.

The respiratory rate often was depressed, in some instances falling to as low as four respirations per minute. At that stage, respiration was assisted manually. When the rate is at 12 respirations per minute, ventilation is adequate, as the depth of respiration seems to be increased with oxymorphone. There was no clinical evidence of overaccumulation of carbon dioxide. At the end of the operation, if the respiratory rate was inadequate, an intravenous injection of nalorphine hydrochloride, ½ to 1 ml. (2.5 to 5 mg.), brought about an immediate response to normal.

No histamine reactions were observed.

*'Vasoxyl,' Burroughs Wellcome & Co. (U.S.A.) Inc.

Discussion

"Balanced anesthesia," a term coined by Lundy,[5] has been used extensively to designate the triad of the state of anesthesia—amnesia, analgesia, and relaxation. In the years when diethyl ether was the sole anesthetic agent used, all the individual prerequisites of anesthesia were supplied by the single agent. With the advent of thiopental sodium, the amnesia it produced could be supplemented by analgesic agents such as nitrous oxide, halothane, or cyclopropane. Relaxation was produced by curare derivatives or succinycholine chloride, but all the anesthetic superstructure was built on a foundation of narcotic premedication. In many departments of anesthesiology the policy is to use minimal premedication. Now it becomes necessary for the anesthesiologist to supplement the premedication by the intravenous injection of an additional narcotic. In trials of various narcotics, the useful effects of oxymorphone were discovered.

Numerous technics using various agents were attempted before the method described was evolved. Early in this study, halothane and divided doses of meperidine were employed to provide general anesthesia (amnesia and analgesia). Halothane was particularly useful, as it causes bronchodilatation. It was the preferred agent in those patients with respiratory disease, but the use of epinephrine by the surgeon incurred the possibility of inducing aberrant myocardial rhythms, and necessitated a revision of the anesthetic technic. Although halothane offers ideal analgesic qualities for surgical procedures on the head and neck, it has the one great disadvantage of sensitizing the heart to epinephrine. The inhalation of halothane, therefore, in our series was replaced by inhalation of nitrous oxide and oxygen. To supply sufficient analgesia with the nitrous oxide—oxygen technic, additional basal narcosis was required. Meperidine as a supplement was ineffective, and in adequate doses produced hypotension. In view of this experience it was decided to assess the value of oxymorphone.

It was immediately evident that when oxymorphone was administered during anesthesia, it produced a profound analgesic effect. In fact, the effect was much greater than that produced in a patient not under general anesthesia. For this reason, we are led to believe there is a synergistic effect between the oxymorphone and the nitrous oxide, thiopental sodium, or a combination of nitrous oxide and thiopental sodium. The amount of oxymorphone for basal narcosis must be reduced as compared to the amount administered in the preoperative and postoperative periods. The patient's respiratory excursions now are easily controlled. This is a great advantage when inducing anesthesia for head and neck operations; the patient must not cough or strain.

The synergism with the other anesthetic agents may also cause the decline in respiratory rate. However, associated with this reduction is an increase in tidal volume. Spontaneous respiratory excursions during such surgical procedures are supported by manual compression of the breathing bag or by an artificial respirator. When necessary, the respiratory rate may be returned to normal by the intra-

venous injection of nalorphine, as mentioned.

In some patients the blood pressure was so altered as to cause a hypotension in the range of 10 to 20 mg. of Hg. This range in most cases is within the normal physiologic bounds. It contrasts greatly with the circulatory depression experienced with the administration of meperidine, after which the blood pressure often verges on levels of shock. The hypotension caused by oxymorphone can be rapidly reversed, if necessary, by the administration of any vasopressor.

Summary

The problems in anesthetizing patients who are to undergo operations on the head and neck include coping with already existing pulmonary insufficiency; and keeping the anesthesia as light as feasible to allow prompt recovery after long surgical procedures. Oxymorphone hydrochloride has given profound analgesia, and good operating conditions for the surgeon in a series of 50 patients. The drug was safe as well as effective. Unlike other narcotic agents, oxymorphone does not produce deep hypnosis, but rather a state of profound tranquility and freedom from pain. The patient may be aroused easily and controls all his vital reflexes.

References

1. Blumberg, H., and Dayton, H. B.: High activity of potent analgesics on conditioned rat tranquilizer test. Proc. Soc. Exper. Biol. & Med. **101:** 594-596, 1959.

2. Coblentz, A., and Bierman, H. R.: Analgesic properties of Numorphan (14-hydroxy dihydromorphinone); new synthetic narcotic. New England J. Med. **255:** 694-698, 1956.

3. Samuels, M. L.; Stehlin, J. S.; Dale, S. C., and Howe, C. D.: Critical evaluation of Numorphan: new synthetic morphine-like alkaloid. South. M. J. **52:** 207-210, 1959.

4. Eddy, N. B., and Lee, L. E., Jr.: Analgesic equivalence to morphine and relative side action liability of oxymorphone (14-hydroxydihydromorphinone). J. Pharmacol. & Exper. Therap. **125:** 116-121, 1959.

5. Lundy, J. S.: Clinical Anesthesia: A Manual of Clinical Anesthesiology. Philadelphia: W. B. Saunders Co., 1942, 771 pp.

EVALUATION OF THE REITER PROTEIN COMPLEMENT-FIXATION (RPCF) TEST FOR SYPHILIS*

JEROME J. BERNER, M.D.,† JOHN W. KING, M.D., Ph.D.,
and ALFRED REICH, B.S.
Department of Clinical Pathology

IN recent years many new serologic tests have been developed for the diagnosis of syphilis. Some of these tests are so complex as to be restricted to research institutions or large public health laboratories. The Reiter protein complement-fixation (RPCF) test, however, can be readily performed in a small hospital laboratory and, according to most published reports,[1-3] the results compare favorably with those of the more expensive and difficult *Treponema pallidum* immobilization (TPI) test for syphilis. For these reasons, it seemed desirable to evaluate the RPCF test along with the cardiolipin procedures that have been performed as standard procedures in our serology laboratory[4] (Kolmer complement-fixation test since 1921, and Kahn test since 1925).

Historical Background

The evolution of the various serologic tests for syphilis had a rather slow beginning, but has been progressing at great speed during the last decade. Schaudinn and Hoffmann[5] described the *Treponema pallidum* as the etiologic agent of syphilis in 1905, and in the following year a serologic test for syphilis was reported by Wassermann, Neisser, and Bruck,[6] who, using a complement-fixation procedure, employed saline extracts of organs containing many treponemata as antigens. In 1907, serviceable antigens were prepared from normal tissues, and by 1911 several antigens had been derived from alcoholic extracts of beef heart muscle plus cholesterol. The quality of lipoidal antigens has been improved by Pangborn's[7] isolation of cardiolipin (a phospholipid from beef heart), the development of methods for the purification of lecithin from beef heart and egg yolk, and the use of synthetic lecithin.

A great departure from the lipoidal antigens was the use of the living, virulent, Nichols strain of *Treponema pallidum* in the TPI test developed by Nelson and Mayer[8] and reported in 1949. Since that time, there has been a spate of diagnostic procedures employing various antigens derived from intact or chemically fractionated virulent (Nichols) or avirulent (Reiter) strains of *Treponema pallidum.* Garson[9] classified the various tests on the basis of derivation of their antigens. We have amplified his outline as follows.

**Read by title at the meeting of the Society of American Bacteriologists, Philadelphia, Pennsylvania, May 1-5, 1960: Berner, J. J.; Reich, A., and King, J. W.: (Abs.) Clinical evaluation of Reiter protein complement fixation test. Analysis of 3122 parallel serologies. Bact. Proc.: 143, 1960.*

†Formerly Fellow in the Department of Clinical Pathology; present address: Barberton Citizens Hospital, Barberton, Ohio.

I. Those tests using whole-body virulent *Treponema pallidum* (Nichols strain) as antigen
   A. Tests using viable organisms from rabbit syphilomas
      (1) *Treponema pallidum* Immobilization (TPI): positive test consists in the demonstration under the dark-field microscope of immobilization of motile treponemata by syphilitic serum.
      (2) *Treponema pallidum* Methylene Blue (TPMB): performed as the TPI test except that the motile organisms take up the methylene blue dye and are more easily seen.
   B. Tests using, or usually using, nonviable organisms
      (1) *Treponema pallidum* Agglutination (TPA): poorly reproducible; detects reagin as well as treponemal antibody.
      (2) *Treponema pallidum* Immune Adherence (TPIA): in presence of syphilitic antibody and complement, killed organisms adhere to human erythrocytes and are absent from the supernatant following centrifugation.
      (3) Whole-Body *Treponema pallidum* Complement Fixation (WBTPCF): uses TPIA antigen in Kolmer-type complement fixation.
      (4) Fluorescent Treponemal Antibody (FTA): syphilitic antibody attaches to dried treponeme suspension, which in turn binds fluorescein-tagged human antiglobulin.

II. Those tests using a chemical fraction derived from whole-body virulent *Treponema pallidum* (Nichols strain) as antigen
   A. *Treponema pallidum* Complement Fixation (TPCF): employs aqueous desoxycholate extract of organisms; modification[10,11] as "tpcf 50" test increases speed and decreases cost of test.
   B. Treponemal Wassermann Reaction (TWR): antigen derived from mechanically disintegrated organisms and used in complement-fixation procedure.
   C. *Treponema pallidum* Cryolysis Protein Reaction (TPCPR): antigen derived by cycles of freezing and thawing.

III. Those tests using a chemical fraction derived from whole-body Reiter treponeme as antigen
   A. Reiter Protein Complement Fixation (RPCF): antigen prepared by ultrasonic or cryolytic disruption of organisms, precipitation with ammonium sulfate, followed by dialysis.
   B. Kolmer Test with Reiter Protein Antigen: as above.

The most recent advance in the serodiagnosis of syphilis is the rapid plasma reagin (RPR) test that employs unheated plasma[12] or serum,[13] and affords test results within 10 minutes from the time the blood specimen is drawn from the patient.

Most of the tests outlined above cannot be performed in routine serology

laboratories because of the complexity of the procedures, the danger of handling virulent organisms, the great amount of time and expense involved, and the difficulty in obtaining many of the antigens. None of these problems apply to the performance of the RPCF test.

The Reiter strain of *Treponema pallidum* was isolated in 1922 by Wassermann and Ficker[14] and, according to Sequeira,[15] subsequently studied by Reiter. This strain is distinctive in that it is avirulent, it can be cultured on laboratory media (Brewer's thioglycollate broth), and furthermore it can survive long periods without subculture. Antigens comprised of suspensions of intact organisms have been used in Germany since 1939, but were unsatisfactory because a lipid component in the treponemal antigen resulted in many nonspecific cross reactions with the standard Wassermann tests employing lipoidal antigens. In 1953, D'Alessandro and Dardanoni[16] isolated from the Reiter treponeme a protein, a carbohydrate that is relatively unimportant, and two lipid fractions, one of which is responsible for the nonspecific Wassermann cross reactions. The thermolabile protein antigen is prepared by the disruption of the treponemes by cryolysis or by ultrasonic waves and subsequent precipitation with ammonium sulfate, followed by dialysis. This protein antigen of the Reiter treponeme has been shown[17] to be immunologically identical with a protein fraction similarly derived from the virulent Nichols strain of *Treponema pallidum.* In addition to this common antigen, the virulent strain is also believed[18] to have a specific component that is lacking in the Reiter organism.

Material and Methods

In order to evaluate the RPCF test in parallel with the cardiolipin procedures, we conducted a comparative study in the following manner. Beginning in October, 1959, all serums received in the serology laboratory were tested by the qualitative Kolmer, Kahn, and RPCF tests. Each serum that showed a four-plus reaction was retested quantitatively with the antigen or antigens with which it reacted, in five-tube serial dilutions in the case of the RPCF and Kolmer tests, and in eight-tube serial dilutions for the Kahn test.

The Reiter protein complement-fixation test is performed according to standard complement-fixation technics, employing one-fifth quantities of reagents and using the Reiter protein antigen* according to the procedure outlined by the Venereal Disease Experimental Laboratory.[1] The Kolmer complement-fixation test is performed with Kolmer's cardiolipin antigen.† The three-tube Kahn test followed the accepted Kahn technic, using the standard Kahn antigen‡.

Results

During the four and one-half month period encompassed by this study, 10,292

**Commercially prepared by the Sylvana Chemical Company, and Organon, Inc.*

†Commercially prepared by the Sylvana Chemical Company.

‡Prepared in Doctor Kahn's laboratory at the University of Michigan.

serums were tested by all three procedures in the manner outlined. Of this group, 220 specimens reacted positively to at least one of the tests *(Table 1)*. The patients categorized as definitely having syphilis had well-documented clinical histories of this disease. The patients who had histories strongly suggestive of syphilis were

Table 1.—*Analysis of 220 specimens of serum having positive serologic reactions*

Tests	Category, number of patients				
	Syphilis		BFP reactors	Incomplete clinical data	Total
	Definite	Probable			
RPCF	3	13	8	27	51
RPCF, Kolmer, and Kahn	32	5	2	8	47
Kolmer	4	3	13	25	45
Kolmer and Kahn	12	2	9	9	32
Kolmer and RPCF	13	3	1	14	31
Kahn	0	1	7	5	13
Kahn and RPCF	0	0	0	1	1
Total	64	27	40	89	220

referred to as probably syphilitic. The patients classified as biologic false-positive (BFP) reactors had no clinical evidence of syphilis or history suggestive of the disease. Eighty-nine patients had no clinical evaluation of their syphilitic status at the Cleveland Clinic, either because of their return to their referring physicians, or the imperative nature of the underlying disease. These serums were not included in the final clinicoserologic evaluation of the tests for syphilis which we studied. The serums of 131 patients were retained for this evaluation. The 220 positive specimens reacted with the several serologic tests in the distribution outlined in *Table 1*. One-half of all patients who were classified as definitely having syphilis, reacted positively to all three of the serologic tests. Among the false-positive reactors the greatest number of reactions, 13, occurred with the Kolmer test; however, eight occurred with the RPCF test, and two patients reacted to all three of the serologic tests. A curious finding is the occurrence of only one positive reaction, among all clinical categories, to the combination of Kahn and RPCF tests. More serums reacted with the Kolmer antigen than with the Kahn or RPCF antigens. This high degree of sensitivity has long been known, and accounts for the many biologic false-positive reactions given by the Kolmer test.

Our study was conducted in a manner similar to that of Foster, Nicol, and Stone,[19] of St. Thomas' Hospital, London, and our two series are compared in *Table 2*. Seven times as many patients examined at the Cleveland Clinic had negative RPCF tests as those examined at St. Thomas' Hospital. The following hypotheses are suggested to explain this difference. The particular group of

Table 2.—*Comparison of patterns of reaction to standard serologic tests for syphilis and to the Reiter protein complement-fixation (RPCF) test*

Test combination	Series, per cent	
	Cleveland Clinic (220 cases)	St. Thomas' Hospital[18] (288 cases)
Positive STS* and positive RPCF	35.8	61.8
Positive STS* and negative RPCF	41.0	4.5
Negative STS† and positive RPCF	23.2	33.7

*Positive STS refers to either Kolmer or Kahn tests.

†Negative STS refers to both Kolmer and Kahn tests.

patients reported by the British authors might be comprised of more syphilitic persons. This possibility is further suggested by the greater number of these patients who had positive reactions to both the standard and Reiter tests for syphilis. In our experience, this pattern of reactivity to both the lipoidal and treponemal tests was most often encountered among patients who were considered clinically to be definitely syphilitic *(Table 3)*. Another suggestion to explain

Table 3.—*Clinicoserologic evaluation of 131 patients*

Test combination	Clinical category, number of patients		
	Definitely syphilitic	Probably syphilitic	Probably BFP
Positive STS* and positive RPCF	45	8	3
Positive STS* and negative RPCF	16	6	29
Negative STS† and positive RPCF	3	13	8
Total	64	27	40

*Positive STS refers to either Kolmer or Kahn tests.

†Negative STS refers to both Kolmer and Kahn tests.

the lesser numbers of RPCF reactors among the Cleveland Clinic patients could be that the widespread use of antibiotics by physicians in this country might have altered individuals' reactivity to the reagin and treponemal antigen tests.

Table 3 summarizes the clinicoserologic evaluation of the 131 patients in our series for whom there were adequate clinical data. It is apparent that most cases of syphilis were detected by all three serologic tests, and that the largest number of the BFP reactors were detected by the cardiolipin tests and not by the treponemal antigen. The 16 patients with syphilis in whom there were negative reactions to the RPCF antigen represent cases of long duration in which the

Reiter antibody became negative before the reagin did. Included in this group are seven cases in which spinal fluid serologic tests were positive, and three cases of congenital syphilis. The biologic false-positive RPCF reactions occurred in a group of 11 patients *(Table 4)* none of whom had a proved clinical history of syphilis, and all of whom had negative serologic tests for syphilis, either prior to the current study or upon repeated testing during the study. This group of false-positive reactors included six males and five females, whose ages ranged from 7 years to 77 years, and who had few clinical features in common. The major diagnoses of these patients were: demyelinization of the central nervous system, intervertebral disk, mesenteric lymphadenitis, traumatic paraplegia, benign enlargement of the prostate, renal hypertension, carpal tunnel syndrome, arteriosclerosis obliterans, pruritus ani, rheumatoid arthritis, and rheumatic heart disease. There were no clinical or therapeutic factors to account for the false-positive reactions in patients 1, 5, 8, 9, and 11. Patients 9 and 11 reacted positively to all three serologic tests. Patient 2 had malaria in 1941 and questionable syphilis in 1944. Patient 3 had positive serologic reactions during a period in which he was suspected of having an abdominal aneurysm; at laparotomy, intestinal adhesions and mesenteric lymphadenitis were found. Patient 4 had the false-positive RPCF reaction within a few days of a cerebral infarction. Patient 6 had hypertension associated with renal artery stenosis. Patients 7 and 10 had rheumatoid arthritis, which is reported to be often associated with false-positive serologic tests for syphilis. The occurrence of false-positive reactions to the treponemal antigen might suggest that perhaps all of the nonspecific lipoidal components had not been completely removed or that some of the patients actually had undiagnosed syphilis.

In order to obtain a rough comparison of the relative performance of these tests, we may calculate the specificity, sensitivity, and BFP rate of each individual test and each combination of tests. The specificity of a serologic test for syphilis is defined as the percentage of nonreactive results obtained in a nonsyphilitic population. For this group we selected the blood donors from the Cleveland Clinic blood bank. This particular group had been previously surveyed by King and Reich[4] who found that among 12,000 blood donor samples examined during a seven-year period, three cases of syphilis were discovered by the routine (Kolmer and Kahn) serologic survey. This gave an incidence of 0.025 per cent for this particular group. In our current study, among the 10,292 specimens of serum tested, 1058 were from professional donors in our blood bank. Of this group, three *previously negative* donors reacted positively as follows:

	Test, reaction		
Donor	Kolmer	Kahn	RPCF
A	+	−	−
B	−	−	+
C	−	−	+

We have assumed this to represent a lack of specificity of the particular tests, but have subtracted 0.025 per cent from the percentage of positive reactors (as representing the random chance that they might have actually had syphilis) to derive the specificity of each test.

The sensitivity of a serologic test for syphilis is defined as the percentage of

Table 4.—*Analysis of data of 11 patients who were biologic false-positive reactors to the RPCF test*

Patient, no.	Age	Sex	Test, reaction			Date
			Kolmer	Kahn	RPCF	
1	46	M	Neg.	Neg.	—	7/16/54
			Neg.	Neg.	—	10/13/58
			Neg.	Neg.	32200	11/19/59
			Neg.	Neg.	Neg.	1/19/60
			*Neg.	Neg.	Neg.	1/22/60
2	52	M	Neg.	Neg.	30000	11/24/59
			Neg.	Neg.	Neg.	12/8/59
3	56	M	Neg.	Neg.	Neg.	12/16/59
			Neg.	Neg.	44210	1/4/60
			Neg.	Neg.	44200	1/11/60
4	50	F	Neg.	Neg.	—	8/4/58
			*Neg.	Neg.	—	9/5/58
			Neg.	Neg.	44422	1/7/60
5	77	M	Neg.	Neg.	42200	1/11/60
6	42	M	Neg.	Neg.	Neg.	12/15/59
			Neg.	Neg.	44300	1/15/60
7	49	F	Neg.	Neg.	Neg.	12/1/59
			44443	Neg.	44200	1/15/60
			Neg.	Neg.	Neg.	2/16/60
8	65	M	Neg.	Neg.	31000	1/24/60
			Neg.	Neg.	Neg.	2/4/60
9	7	F	44321	2+	21000	1/25/60
			2+	Neg.	†AC	2/1/60
10	37	F	Neg.	Neg.	41000	1/25/60
			Neg.	Neg.	Neg.	2/10/60
11	57	F	Neg.	Neg.	—	7/21/59
			32210	2+	20000	2/24/60
			10000	2+	Neg.	3/1/60

*Cerebrospinal fluid specimen.
†Anticomplementary.

reactive results among a group of known syphilitic persons. The group of 64 patients classified as definitely syphilitic was used in this calculation.

A third expression of evaluation of these tests (suggested by Wheeler) is that of the BFP rate,[20] which is the percentage of reactive results among those patients whose previous serologic and clinical histories suggest a diagnosis of BFP sero-

Table 4.—*Concluded*

Patient, no.	Diagnosis	Remarks
1	Demyelinization of central nervous system; mechanical back pain.	
2	Protruded lumbar disk	Malaria in 1941; treated in 1944 for syphilis (?) without positive serologic test.
3	Mesenteric lymphadenitis	Laparotomy 1/7/60; hospital discharge 1/14/60.
4	Traumatic paraplegia; essential hypertension; cerebral infarction 1/4/60.	
5	Benign enlargement of prostate	
6	Renal hypertension; cerebral artery thrombosis	Ten negative Kolmer and Kahn tests since 1945 Splenoreal anastomosis 1/18/60; died 1/29/60.
7	Carpal tunnel syndrome; rheumatoid arthritis.	
8	Arteriosclerosis obliterans of femoral artery; dermatophytosis	
9	Pruritus ani	Mother's serologic test negative
10	Rheumatoid arthritis	Treated with ACTH, nitrogen mustard, and chloroquine phosphate 2/6/60.
11	Rheumatic heart disease; mitral insufficiency.	

logic reactions. The group of 40 patients classified as probable BFP reactors was used in this calculation.

The serologic tests listed in *Table 5* are in the order of their over-all performance. It is apparent that the performance value of a serodiagnostic test for syphilis

Table 5.—*Comparison of serologic tests (listed in order of over-all performance)*

Tests	Measurement of performance, per cent		
	Specificity	Sensitivity	BFP rate
Kolmer and RPCF	99.7	70.0	7.5
RPCF	99.8	75.0	27.5
RPCF and Kahn	99.8	50.0	5.0
Kolmer and Kahn	99.9	68.7	27.5
Kolmer	99.9	95.3	62.5
Kahn	100.0	68.7	45.0

is best reflected by a combination of the three parameters of specificity, sensitivity, and BFP rate. To consider any one of these parameters exclusively, will give a misleading impression of the value of the various test procedures. Furthermore, a different test or combination of tests excels in each of the categories. In our experience, the single most specific procedure was the Kahn test, the single most sensitive was the Kolmer test, and the combination resulting in the least number of BFP reactions was the RPCF and Kahn tests. In considering all three categories together, we have found that the best diagnostic procedure is to combine the Kolmer and the RPCF tests. This observation is consistent with the recommendations of other groups of investigators that a combination of a reagin and a treponemal test is better than either test alone, because each measures a different antibody and is a complementary procedure.[15]

Discussion

Since publication of the first evaluations of the RPCF test by DeBruijn[2] in Holland, and Cannefax and Garson[1] in the United States, many workers have reported the close correlation of the RPCF test with the TPI procedure. As regards specificity, these workers and the many institutions which participated in the Serology Evaluation and Research Assembly[20] (SERA) reported the RPCF to have a range of specificity from 94.4 to 97.7 per cent. This compares favorably with the TPI test, which has a reported specificity of 93.7 to 100.0 per cent. In DeBruijn's[2] series, a standard serologic test for syphilis, which uses a cardiolipin antigen, gave a specificity of 82 per cent; the various nontreponemal tests in the SERA[20] study gave specificities ranging from 83 to 99.5 per cent.

The sensitivity of the various serologic tests for syphilis is a function of the

particular stage of the disease *(Fig. 1)*. In its earliest stages the disease is seldom detected with the TPI test, but is more readily diagnosed with the various nontreponemal and Reiter tests. However, in the late stages of syphilis, the TPI test remains positive long after the reagin and RPCF tests have become negative. In

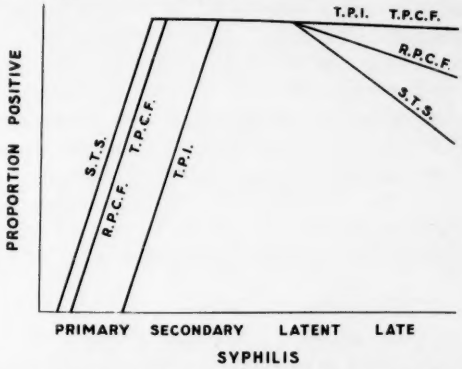

Fig. 1. Sensitivities of certain serologic tests at different stages of syphilis. (Courtesy of Sequeira, P. J. L.: Brit. J. Vener. Dis. 35: 141, 1959; and the Editor and Publishers of the *British Journal of Venereal Diseases,* London, England.)

its over-all sensitivity, the RPCF is reported[20] to surpass the TPI test and to be more sensitive than some, but not all, of the various nontreponemal tests.

In the SERA[20] report, Wheeler compares the BFP rate of the various types of tests. The RPCF and TPI procedures are reported to give ten times fewer BFP reactions than the various nontreponemal tests. The patients comprising this group were selected on the basis of previous clinical and serologic histories which along with a negative TPI suggested a diagnosis of BFP. In contrast to our 27.5 per cent BFP rate for the RPCF test, Wheeler found only 4 per cent of this group of patients to react to the RPCF test. Most observers believe that the RPCF test has its greatest usefulness in the differentiation of BFP reactors.

In view of the many false-positive reactions to the RPCF test in our personal experience, we believe that unqualified acceptance of the RPCF test results is not warranted, but that they should be checked further with the TPI test. One such study of discrepant RPCF and reagin results, which were then further checked by means of the TPI test, was conducted by Foster, Nicol, and Stone[19] in cooperation with Dr. P. J. L. Sequeira of the Central Serological Laboratory, Manchester, England. Of 150 samples of serum that showed discrepant reactions between a standard microflocculation test and the RPCF test, 20 per cent showed disagreement also between the results of the RPCF and the TPI tests. Most of these serums reacted negatively to the TPI and positively to the RPCF test. The authors suggest that these might have been cases of early stages of syphilis in which the TPI test result had not yet become positive; we suggest that some of their cases might just

as well have represented false-positive RPCF reactions. They also found that in a group of patients upon whom they performed repeated RPCF tests with successive serum samples, results were variable in 19 per cent. Almost half of their patients who gave variable RPCF results had positive results in TPI tests, suggesting to the authors that even a variable RPCF test could indicate a syphilitic infection. In our own series we found a 9.5 per cent variability rate, which was identical for the RPCF, Kolmer, and Kahn tests. Our belief is that one should not rely solely upon any single laboratory finding, particularly when successive serums from the same patient give varying results. The danger inherent in dependence upon the RPCF test alone, is evidenced in a comparative study[19] in which 132 serums were independently tested by two laboratories. Identical results were obtained in only 99 of the specimens. These findings further suggest that there is too much variability in the results of the RPCF test for this procedure to be used as the sole or ultimate criterion in the serodiagnosis of syphilis.

Conclusions

1. According to our experience the best testing method in the serodiagnosis of syphilis is the combination of the Kolmer test and the Reiter protein complement-fixation (RPCF) test.

2. The majority of patients with syphilis reacted positively to both serologic tests.

3. The RPCF test, alone, gave far more biologic false-positive (BFP) reactions than have been reported by other investigators using this test.

4. The diagnosis of syphilis should not be based upon a positive RPCF test alone.

5. To avoid error:

   All serologic test results should be interpreted in relation to a detailed clinical history.

   A serologic test that gives a positive result should be repeated.

   If the test result is at variance with the clinical impression, the result should be rechecked, first using the RPCF test, and subsequently, if indicated, the *Treponema pallidum* immobilization (TPI) test.

Acknowledgment

The authors wish to express their sincere appreciation to Mrs. Esther Travinski and Mrs. Patricia Blumel of the Serology Laboratory of the Department of Clinical Pathology for the performance of the laboratory procedures included in this study.

References

1. Cannefax, G. R., and Garson, W.: Reiter protein complement fixation test for syphilis. Public Health Rep. 72: 335-340, 1957.

2. DeBruijn, J. H.: Application of protein fraction derived from *Treponema pallidum* (Reiter strain) as antigen in serodiagnosis of syphilis. Antonie Van Leeuwenhoek 23: 201-206, 1957.

3. Rein, C. R.; Kelcec, L. C.; D'Alessandro, G., and DeBruijn, J. H.: Sensitivity and specificity of Reiter protein complement fixation (RPCF) test for syphilis. J. Invest. Derm. **28:** 459-462, 1957.

4. King, J. W., and Reich, A.: Usefulness of routine serologic tests for syphilis. Cleveland Clin. Quart. **24:** 174-180, 1957.

5. Schaudinn, F., and Hoffmann, E.: Vorläufiger Bericht über das Vorkommen von Spirochaeten in syphilitischen Krankheitsprodukten und bei Papillomen. Arb. a.d. Kaiserl. Gesundh. **22:** 527-534, 1905.

6. von Wassermann, A.; Neisser, A., and Bruck, C.: Eine serodiagnostische Reaktion bei Syphilis. Deutsche med. Wschr. **32:** 745-746, 1906.

7. Pangborn, M. C.: New serologically active phospholipid from beef heart. Proc. Soc. Exper. Biol. & Med. **48:** 484-486, 1941.

8. Nelson, R. A., Jr., and Mayer, M. M.: Immobilization of Treponema pallidum in vitro by antibody produced in syphilitic infection. J. Exper. Med. **89:** 369-393, 1949.

9. Garson, W.: Recent developments in laboratory diagnosis of syphilis. Ann. Int. Med. **51:** 748-758, 1959.

10. Bossak, H. N.; Duncan, W. P.; Harris, A., and Falcone, V. H.: Evaluation of tpcf-50 and other TPCF tests for syphilis. Public Health Rep. **75:** 130-133, 1960.

11. Portnoy, J.: Complement-fixation with small volumes of reagents; application to *Treponema pallidum* complement-fixation test for syphilis (tpcf 50). Am. J. Clin. Path. **31:** 316-322, 1959.

12. Simpson, W. G.; Matthis, A. W.; Harris, A., and Price, E. V.: Evaluation of rapid plasma reagin test in field operation. Public Health Rep. **74:** 473-477, 1959.

13. Portnoy, J., and Garson, W.: Preliminary report on RPR test for syphilis using unheated serum. Public Health Rep. **74:** 965-968, 1959.

14. von Wassermann, A., and Ficker, M.: Reinkulturen der Spirochaete pallida in festem und flüssigem nährboden, sowie Übertragung dieser Kulturen auf Tiere. (Inoculation of rabbits by pure cultures of spirochaeta pallida.) Klin. Wschr. **1:** 1101, 1922.

15. Sequeira, P. J. L.: Examination of treponemal Wassermann reaction and Reiter protein complement-fixation test. Brit. J. Vener. Dis. **35:** 139-147, 1959.

16. D'Alessandro, G., and Dardanoni, L.: Isolation and purification of protein antigen of Reiter treponeme; study of its serologic reactions. Am. J. Syph. **37:** 137-150, 1953.

17. Cannefax, G. R., and Garson, W.: Demonstration of common antigen in Reiter's treponeme and virulent *Treponema pallidum.* J. Immunol. **82:** 198-200, 1959.

18. Wilkinson, A. E., and Johnston, N. A.: Results of parallel tests with Reiter protein complement fixation test, treponemal immobilization test, and treponemal Wassermann reaction on 1,046 sera. Brit. J. Vener. Dis. **35:** 175-180, 1959.

19. Foster, W. D.; Nicol, C. S., and Stone, A. H.: Further assessment of Reiter protein complement-fixation (RPCF) test in routine serological laboratory. Brit. J. Vener. Dis. **35:** 181-183, 1959.

20. U. S. Department of Health, Education, and Welfare, Public Health Service: Serology Evaluation and Research Assembly; 1956-1957. Public Health Service Pub. No. 650. Washington, D.C.: U. S. Gov't. Printing Office, 1959, 214 pp.

METHOXYFLURANE* — A NEW ANESTHETIC AGENT

A Clinical Evaluation Based on 206 Cases

CARL E. WASMUTH, M.D., LL. B., JOHN H. GREIG, M.D.,†
JOHN HOMI, M.D.,† PATRICK P. MORACA, M.D., NIYAZI H. ISIL, M.D.,†
ELMARS M. BITTE, M.D., and DONALD E. HALE, M.D.
Department of Anesthesiology

METHOXYFLURANE, the most satisfactory anesthetic agent of a series of fluorinated hydrocarbons and fluorether studied clinically by Artusio and Van Poznak,[1] was used by us to induce or to maintain anesthesia in 206 patients. This anesthetic agent is a nonexplosive liquid that produces profound analgesia accompanied by remarkable muscular relaxation with apparently low toxicity. In low vapor concentrations, methoxyflurane produces the same degree of analgesia produced by halothane, the same degree of muscular relaxation produced by deep levels of cyclopropane anesthesia, and possesses the same wide margin of safety as that of diethyl ether.

Introduced experimentally as DA-759, methoxyflurane is 1,1-dichloro-2, 2-difluoro-2-methoxyethane. The chemical structure is shown in *Figure 1.* It is a

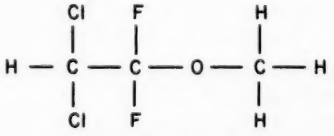

Fig. 1. Structural formula for methoxyflurane.

clear, colorless liquid that boils at 104.8 C. (220 F.) ± 0.2 degrees at 760 mm. of Hg, and has a specific gravity of 1.4224. The odor is pleasant with a fruity characteristic. The explosive range is shown in *Table 1.* At 20 C. (68 F.) the explosive limits in air and oxygen are zero. The flash point is 56.11 C. (133 F.). The vapor density at 37 C. (98.6 F.) is 7.36 gm. per liter. It is miscible in all proportions with olive oil (liquid to liquid). Its solubility in water, according to polarographic titration is 0.22 gm. per liter of water.

The olive oil/water distribution coefficient was determined for 1 per cent methoxyflurane as 400. Using a 10 per cent concentration of methoxyflurane, the distribution constant was 39. Diethyl ether is 3.8 and halothane is 330. *Table 2* lists the vapor pressures of methoxyflurane.

The absolute viscosity at 20 C. is 1.070 and at 50 C. is 0.703 centipoise. The surface tension at 20 C. is 25.55 and at 50 C. is 22.61 dynes per centimeter. The

**DA-759, commercially available as Penthrane, Abbott Laboratories.*
†Fellow in the Department of Anesthesiology.

Table 1.—*Explosive range of methoxyflurane*

Methoxyflurane mixed with	Percentage, per cent	Temperature C.	Temperature F.	Percentage, per cent	Temperature C.	Temperature F.
Air	9	80	176	28	105	221
Oxygen	4	60	140	28	105	221

Table 2.—*Vapor pressures of methoxyflurane*

Pressure, mm. of Hg	Temperature, C.
665.06	100.15
558.94	94.78
459.78	88.78
188.93	64.52
90.29	47.24
40.0	29.0*
30.0	24.4*
10.0	5.0*

*Extrapolated value.

ultraviolet-absorption spectra and infrared-absorption spectra of methoxyflurane were studied by Chenoweth.[2]

With the Vernitrol (Heidbrink) vaporizer the amount of methoxyflurane vaporized was measured during various flow rates of 100 to 1000 ml. through the vaporizer. The temperature of the vaporizer was 23 C. (74 F.) for all the determinations. Each determination was made by replacing the measured volume of methoxyflurane; 1 ml. of liquid forms 209.7 of vapor.

Table 3 shows the percentages of vaporization with the various flow rates. Because the temperature of liquid methoxyflurane in the vaporizer remained constant at the higher flow rates, at 23 C. an average of 3.7 per cent methoxyflurane vapor resulted from 26 ml. of oxygen in the vaporizer and 1 ml. of methoxyflurane. By extrapolation, the vapor pressure of methoxyflurane (for 30 minutes at 24 C. and 28 ml. of oxygen flow through the vaporizer) is 1 ml. of methoxyflurane vapor for a vapor percentage of 3.57 per cent. Thus, our percentage of vaporization, according to average determinants is close to the theoretic value.

The percentage of methoxyflurane vaporized with a Heidbrink ether wick vaporizer was determined for a 4-liter flow at various openings of the vaporizer.

Table 3.—*Vaporization of methoxyflurane at 23 C. in the Heidbrink No. 8 wick vaporizer*

	Flow rate, milliliters per minute		Percentage of methoxyflurane vapor, per cent
	Vaporizer oxygen	Methoxyflurane vapor	
	100	3.78	3.78
	200	10.5	5.25
	300	9.8	3.26
	400	18.2	4.37
	500	18.2	3.52
	600	22.4	3.63
	700	24.5	3.38
	800	30.8	3.71
	900	35.0	3.75
	1000	38.5	3.72
Average	26 ml.	1 ml.	3.7 per cent

This was done by measuring the amount of liquid methoxyflurane vaporized in one hour, and calculating the percentage of vapor on the basis, as previously stated, that 1 ml. of liquid methoxyflurane vaporizes into 209.7 ml. at 23 C. The temperature of the liquid methoxyflurane in the vaporizer varied one degree, from 22 to 23 C. *Figure 2* shows the percentages of methoxyflurane vaporized at various openings of the vaporizer with a 4-liter flow of oxygen.

Metabolic Effects

Since the publication of reports of fatal hepatic damage following chloroform anesthesia, each new substance introduced as a possible anesthetic agent has been evaluated for potential toxic effects on the vital organs and tissues of the body as well as for efficiency in producing anesthesia. It is well established that the members of this group of halogenated hydrocarbons have great potential toxic properties, particularly for the liver. Of the numerous biochemical assays of hepatic function, we used four basic tests on our patients: the tests for blood sugar, Bromsulphalein retention, serum alkaline phosphatase, and serum bilirubin.

Blood sugar assays were made preoperatively to provide a base line in each case, and two additional assays were made during anesthesia, one shortly after induction, the other at the end of the administration of methoxyflurane. The tests were repeated 24 hours postoperatively and at intervals thereafter until the patient was discharged from the hospital.

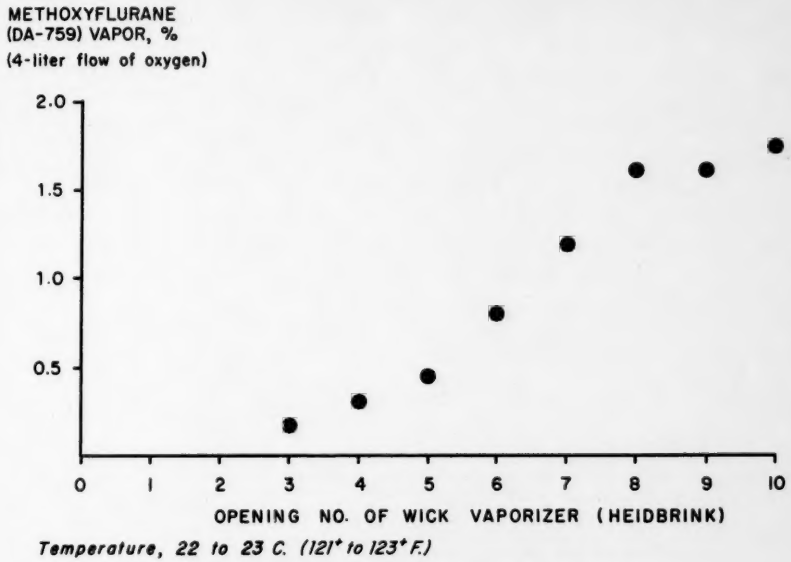

Fig. 2. Chart showing percentages of vaporization of methoxyflurane.

The most striking effect of methoxyflurane was on the blood sugar concentrations. Following induction of anesthesia with methoxyflurane there was a significant rise in the concentration of blood sugar, generally of the order of 10 to 20 mg. per 100 ml. This rise was maintained during the course of anesthesia. *Table 4* shows the values obtained in six patients. The hyperglycemia did not persist postoperatively, despite the fact that all the patients received 5 per cent dextrose intravenously in the immediately and the late postoperative periods. The

Table 4.—*Rise of blood sugar in six patients receiving methoxyflurane anesthesia*

	Blood sugar, mg. per 100 ml.			
Patient no.	Preoperative	Postinduction	At conclusion of operation	Percentage rise, per cent
1	81	95	138	72
2	75	90	118	60
3	70	97	111	57
4	80	93	—	—
5	66	84	92	39
6	72	84	80	11

mode of action of the drug in producing the rise in concentration of blood sugar is presumed to be similar to that described for ether. It is improbable that hypoxia plays a part in this hyperglycemia but, as in the case of agents previously studied, increases in endogenous blood epinephrine, depression of enzymatic activity, blood loss, and sympathetic stimulation cannot be excluded from consideration as factors contributing to this rise.

To date, the small number of patients who have been followed precludes final conclusions as to the effect of methoxyflurane on hepatic function, but the initial impressions seem to be that the impairment of function is comparable to that produced by ether, and that hepatic glycogenolysis with hyperglycemia takes place during anesthesia from methoxyflurane. All patients showed increased retention of the Bromsulphalein in the first 24 hours postoperatively, and gradual return to preoperative values within four or five days.

The alkaline phosphatase and serum bilirubin values were little altered. One patient, a 57-year-old man, however, showed definite alterations in the postoperative values. He was a known alcoholic who had an acute alcoholic exacerbation in the recent past, and it is possible that similar hepatic alterations would have occurred with any other inhalant.

Technic of Administration

Methoxyflurane may be administered in the two ways diethyl ether is administered, by: open-drop technic, or vaporization in a Heidbrink No. 8 wick vaporizer. We used the semiclosed and closed technics. In most instances intubation was employed after using the thiopental sodium—succinylcholine—oxygen sequence. The volume flow of oxygen was usually set at 4 liters, and the vaporizer opening at No. 1 was quickly turned to Nos. 5 or 6. At that flow rate, the patient rapidly became relaxed and anesthetic.

One is immediately impressed with the great amount of muscular relaxation that occurs. Frequently it was sufficient for the entire surgical procedure. But, inasmuch as the amount of relaxation varies with the duration and type of the operation, additional relaxant agents may be required. For example, a great amount of relaxation was needed for procedures such as vagotomy and high resections of the splenic flexure.

Methoxyflurane was administered to induce anesthesia in a few patients. The inhalation of methoxyflurane vapor was somewhat irritating, and the patient coughed during induction of anesthesia. As there were no clinical signs to indicate the level of anesthesia, the stages of anesthesia are difficult to outline. The induction was fairly long, similar to induction of diethyl ether. The excitement stage was short and often was absent. However, the stage of analgesia occurred with exceeding rapidity, especially in the women in labor. Inhaling of the vapor at the time of each successive pain produced a cumulative effect: soon the patient

not only was analgesic but lost consciousness, and the forward progress of the infant's head against the perineum ceased to be painful. However, the analgesia was not equal to the anesthesia necessary to perform surgical procedures, and with application of the forceps, additional amounts of anesthetic agent were required.

The status of the blood pressure is one criterion for judging levels of anesthesia. With deep levels, a fall in blood pressure was noted in many patients, on occasion to 80 mm. of Hg. The hypotension occurred most often when a deeper level of anesthesia was used to obtain additional relaxation. By lightening the anesthesia the hypotension was reversed. Some anesthesiologists contend that additional doses of atropine sulfate will correct this low systolic pressure.

The respiratory pattern in most instances was not altered either in rate or in depth. However, when an automatic respirator was employed, the patient's respirations were easily controlled. When anesthesia was deepened beyond surgical requirements, there were no aberrations in cardiac rhythm or respiratory pattern.

The patient's recovery period, too, was extremely long, as would be expected from the physical characteristics of the anesthetic agent. It was necessary to stop administering the vapor at least from one-half to one hour before the operation was completed. When the vapor was not discontinued in time, the patient did not arouse until the remotely postoperative period. This delay encouraged postanesthetic atelectasis in some of our first patients treated. However, after methoxyflurane anesthesia, a period of analgesia persisted for an extended time into the postanesthetic period. During this time the patient was awake or was easily aroused and was in possession of his vital reflexes.

Postanesthetic Emergence

During the recovery period the patient passes through a phase of regaining his vital reflexes (emergence), followed by the return of his higher reflexes and functions (arousal). Clearly the duration and technic of anesthesia, the nature of the surgical procedure, and the condition of the patient will all play their parts in influencing the results obtained in evaluating an inhalation agent from this point of view. With due regard to these factors, 35 patients were studied by personal observation in the period of emergence, arousal, and the immediately postoperative period.

According to the duration of anesthesia, and the technic employed, the time for emergence ranged from 10 minutes to one and one-half hours. Toward the end of the series, most patients returned from surgery in full possession of their vital reflexes. The period of arousal, which was gauged from the time the patient moved in response to commands or stimuli to the point at which he was fully rational and orientated in time and space, was often prolonged. After physical stimulation or questioning, the patient might lapse into sleep. The average time for full arousal was from one to two hours, and even then, there was a tendency to somnolence in many patients.

The appearance of the patient. The most striking feature to be observed in these patients as they entered the recovery room, was the chalky whiteness of their skin. This applied to all patients except those with pre-existing hyperpigmentation of the skin. The pale skin, however, was warm and dry, with the exception of the fingers and toes of some patients, which were cold and exhibited some peripheral cyanosis of the nail beds. The chalky pallor of the warm dry skin persisted into the period of arousal, being least obvious when the patient was fully awake and alert.

Eye reflexes and movements. The light reflex and consensual reaction were active in all patients who returned from surgery. In those who had not negotiated the stage of emergence the pupil was fixed in the central position. The conjunctival and corneal reflexes were not investigated in order to avoid trauma to the surface of the eye. Lacrimation and return of activity of the lid and eyelash reflexes usually coincided with each other and with the return of eyeball movements. Normal activity of the eye reflex and normal eye movements were not apparent until the swallowing and laryngeal reflexes were active. There were no nystagmus, difficulty with accommodation, or inflammation of the conjunctivae apparent in these patients in the postoperative period.

Vital reflexes. In all patients returned to the recovery room with an endotracheal tube still in situ, because the vital reflexes had not returned, coughing could be evoked by stimulating the carina or trachea with a catheter passed down the endotracheal tube, or by moving the endotracheal tube and irritating the walls of the larynx and trachea. However "spontaneous" coughing did not occur even after the vital reflexes had returned. The laryngeal reflex became active as soon as swallowing and gagging were observed, and after this, eye movements became active.

At the beginning of the series, two patients retained their endotracheal tubes for one hour and one and one-half hours. This emergence was partly due to the duration of the operation, and partly to the technic of administration.

On extubation there was no incidence of laryngospasm in any patient, and mucous secretion was moderate in amount. However there is no inhalation technic in which laryngospasm is unknown, and the use of methoxyflurane will probably prove to be no exception as the series enlarges.

In those patients with an oropharyngeal airway inserted it was found that a gag reflex could be excited by moving the airway about in the mouth, but if left undisturbed the patient retained the airway until emergence was complete. The patient would often reach up and remove the airway himself and then lapse back into sleep. Vomiting and regurgitation did not occur during emergence, but three patients were nauseated and vomited after the return of their vital reflexes.

Patients completed their emergence, therefore, without laryngospasm, coughing, or vomiting, and without evidence of excessive excitement or delirium. No doubt these features will be encountered as more cases are investigated, but it is believed

that the incidence will be lower than with, for example, diethyl ether.

Seven patients had bouts of shivering and shaking of the extremities. These ranged from fine vibrations of the lower jaw and forearms to extravagant jerking movements of the head and limbs, sufficient to shake the bed in one instance.

The stability of the cardiovascular system. As gauged by measurements of the pulse rate and systemic blood pressure at 15-minute intervals until leaving the recovery room, all these patients demonstrated a remarkable cardiovascular stability. Most patients retained their preoperative pulse rates and systemic blood pressure levels, and in those patients returned from surgery with blood pressures below their normal limits, there was no further deterioration.

The adequacy of respiration. Measurements were made of the minute volume and respiratory rate of patients who were sufficiently anesthetized to retain their endotracheal tubes. In all instances an adequate tidal volume for the patient was evident.

As these postoperative patients appeared free from pain, it was necessary to administer an analgesic drug to only seven patients. Although this was advantageous in some respects, it removed all stimulus for movement and encouraged the accumulation of mucous secretions at the lung bases. These patients were deeply asleep. In the more remotely postoperative period, few complications were noted. However, early in the study, there was evidence of pulmonary atelectasis in six patients. One minor inconvenience when methoxyflurane was used as the sole agent was the persistent characteristic odor of the substance in their rooms and about their persons on the day following operation.

It is apparent that in those patients in whom methoxyflurane was used as the sole agent for procedures requiring anesthesia for more than a half hour, emergence was safe, but full arousal was undoubtedly prolonged.

Discussion

Since the concept of balanced anesthesia was introduced, and the discovery of the ultra-fast-acting barbiturates, the skeletal muscle relaxants, and a series of analgesic drugs for intravenous use, intravenous anesthesia has received an impetus with which few inhalation agents have been able to compete.

The search for the perfect inhalation agent has been relentlessly pursued, because, if such an agent were found, all the requirements of perfect general anesthesia could be displayed in a spontaneously breathing patient who could be detoxicated at will, simply by ceasing to administer the inhalation agent. This would be most desirable in prolonged procedures, when the polypharmacy of the intravenous technics is evident as its greatest disadvantage. The fact that respiration must invariably be assisted or controlled with intravenous methods is also undesirable in prolonged procedures.

It is also worth noting that, owing to the multiplicity of electric equipment often required in the modern operating room, any new agent must fulfill the addi-

tional requirement of being nonexplosive. Another unwelcome criterion to be fulfilled by any new agent is the requirement for compatibility with a variety of new chemicals that may have been administered to the patient before or during anesthesia. The incompatibility of diethyl ether with tetraethylthiuram disulfide, and of halothane with ataractics, ganglion-blocking agents, vasopressors, and relaxants, afford examples. It is against this background of problems that any new inhalation agent must be evaluated, and so much more is expected of such drugs now, than was ever hoped for in the past, that of the numerous chemical compounds evaluated in the last 10 years only one, halothane, has qualified for use.

Under the prevailing conditions it would appear that methoxyflurane, which is a nonexplosive inhalation agent is suitable as a supplementary agent to provide analgesia and muscle relaxation mainly in prolonged abdominal surgery. One may reflect that if methoxyflurane had been discovered at the time when ether and chloroform were newly in use, it might have superseded them both and become a byword in anesthesia, but this is hardly likely to occur today.

In the light of our findings it seems that, under the conditions of administration so far investigated, there is a definite but limited place for the use of methoxyflurane as a supplementary agent to provide additional analgesia. When so used with nitrous oxide it achieves some muscle relaxation and thereby reduces the amount of neuromuscular blocking agents required. The fact that it is nonexplosive, and does not react with soda-lime, enables it to be used safely under circumstances when other agents such as cyclopropane or trichlorethylene would be dangerous. Induction was most easily accomplished with a thiopental sodium—succinylcholine—oxygen sequence. It was not always easy to decide whether the patient was at a plane of anesthesia suitable for surgical stimulation. Except for central fixation of the eyeball, the ocular signs, so useful in the diethyl ether anesthesia, were not in evidence. A reliable criterion was the patient's ability to tolerate an airway or endotracheal tube, the onset of muscular relaxation, and the lack of reflex response to painful stimulation.

When used as a supplement to achieve balanced anesthesia, arousal was not delayed. Particularly satisfactory anesthesia was evidenced when methoxyflurane supplemented the thiopental sodium—nitrous oxide—oxygen sequence. Sometimes the addition of muscle relaxants will be necessary with this technic.

When used as the primary agent with air or oxygen, methoxyflurane has little to commend it. The prolonged somnolence outweighs the advantages of prolonged analgesia. We believe, therefore, that, except in infants, who appear to be suitable for induction with this agent, induction by inhalation of methoxyflurane is marred by the long time required, and by the difficulty to judge when it is complete. However, induction was free from the side effect of vomiting, and, although inducting was not so smooth as that with halothane or cyclopropane it was easier than with diethyl ether.

Probably methoxyflurane will find its widest application in abdominal surgery

and gynecologic procedures *(Table 5)*, and possibly in neurosurgery and ophthalmic operations. It may prove suitable for general anesthesia in obstetrics, but this use has not yet been thoroughly investigated. It probably has a wide mar-

Table 5.—*Procedures or operative areas in 206 patients receiving methoxyflurane anesthesia*

Procedure or area	No. of patients
Abdominal	74
Gynecologic	32
Arteriography	26
Perineal	20
Head and neck	14
Thoracic	13
Genitourinary	9
Extremities	7
Other	6
Neurosurgical	5
Total	206

gin of safety, but we cannot assume this when it has been in use for such a short time. It should be remembered that chloroform was used for many years before the first fatality was reported, and it was even many years later that it acquired a perhaps undeservedly bad reputation.

Summary

A new nonexplosive inhalation agent, methoxyflurane (DA-759), has undergone clinical trial in 206 patients, following a preliminary trial by others in 300 patients. It appears to be a useful supplementary inhalation agent, and its properties, clinical characteristics, possible uses and value are discussed, but time alone can determine its place in modern anesthetic practice.

References

1. Artusio, J., and Van Poznak, A.: Personal communication.

2. Chenoweth, M.: Personal communication.

ERRATA

In the April, 1960, issue of this journal, on page 93, the sentence beginning on line 11 should read: *As the intraluminal pressure continues to rise, a localized bulging (syringomyelocele) or splitting (diastematomyelia), followed by rupture (myeloschisis) will occur at the more immature caudal segments.*

On page 94, line 3, the first word should be cranioschisis (not myeloschisis).

RECENT PUBLICATIONS BY MEMBERS OF THE STAFF

AKUTSU, T.; HOUSTON, C. S.,[1] and KOLFF, W. J.: Roller type of artificial heart within the chest: preliminary report. Am. Heart J. **59:** 731-736, May 1960.

BROWN, C. H.: Carcinoma of the pylorus simulating benign duodenal obstruction. Am. J. Digest. Dis. (New Ser.) **5:** 264-273, March 1960.

BROWN, C.H.: The diagnosis of functional gastrointestinal disorders. Psychosomatics **1:** 92-95, March-April 1960.

BROWN, C. H., and MERLO, M.: The clinical importance of antral gastrin; report of a case with persistent marginal ulcer associated with continued secretion of gastrin. Am. J. Digest. Dis. (New Ser.) **5:** 159-167, Feb. 1960.

BROWN, HELEN B., and PAGE, I. H.: Variable responses of hyperlipemic patients to altered food patterns. J. A. M. A. **173:** 248-252, May 21, 1960.

BROWN, HELEN B., and PAGE, I. H.: A physician's guide to dietary serum cholesterol. Heart Bull. **9:** 48-50, May-June 1960.

CRILE, GEORGE, JR.: Personality traits in organic diseases. Psychosomatics **1:** 1-3, Jan.-Feb. 1960.

CRILE, GEORGE, JR.: Endocrine dependency of tumors of the thyroid. Am. J. Surg. **99:** 533-537, April 1960.

CRILE, GEORGE, JR.: The cancer problem: a speculative review of the etiology, natural history, and treatment of cancer. Perspectives **3:** 358-382, Spring 1960.

CRILE, GEORGE, JR.: Papillary Carcinoma of the Thyroid. Chap. 9, pp. 179-184, *in* Clinical Endocrinology. I., edited by E. B. Astwood. New York: Grune & Stratton, Inc., 1960, 724 pp.

CRILE, GEORGE, JR., and SCHNEIDER, R. W.[2]: The Thyroid and Parathyroid Glands. Chap. 13, pp. 317-325, *in* Christopher's Textbook of Surgery, 7th ed., edited by L. Davis. Philadelphia: W. B. Saunders Co., 1960, 1551 pp.

CURTIS, G. H.: Chronic discoid lupus erythematosus. Virginia M. Month. **87:** 119-126, March 1960.

DREYER, B.;[3] AKUTSU, T., and KOLFF, W. J.: Aortic grafts of polyurethane in dogs. J. Appl. Physiol. **15:** 18-22, Jan. 1960.

DUSTAN, HARRIET P.: Treatment of hypertension. Mod. Concepts Cardiovas. Dis. **29:** 585-590, April 1960.

ERNSTENE, A. C.: Dr. Everett Naughtin Collins, F.A.C.P. Bull. Am. Coll. Physi-

[1] *Formerly Research Associate.*
[2] *Formerly Member of the Staff.*
[3] *Formerly Research Associate; present address: Karl Bremer Hospital, Department of Surgery, University of Stellenbosch, Belleville, C. T., South Africa.*

cians **1:** 37, Jan.-Feb. 1960.

GARDNER, W. H.: Speech Pathology, pp. 637-641, *in* Medical Physics, Vol. 3, edited by O. Glasser. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

GARDNER, W. J., and ANGEL, J.: The mechanism of syringomyelia and its surgical correction. Clin. Neurosurg. **6:** 131-140, 1959.

GARDNER, W. J.; McCORMACK, L. J., and DOHN, D. F.: Embryonal atresia of the fourth ventricle; the cause of "arachnoid cyst" of the cerebellopontine angle. J. Neurosurg. **17:** 226-237, March 1960.

GLASSER, OTTO (Editor): Medical Physics, Vol. 3. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

HALE, D. E.: Anesthesia, pp. 23-32, *in* The Cyclopedia of Medicine, Surgery and Specialties; Review Service 1959, edited by G. M. Piersol and others. Philadelphia: F. A. Davis Co., 1959, 964 pp.

HALE, D. E.: Anesthesia, pp. 16-20, *in* Medical Physics, Vol. 3, edited by O. Glasser. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

HEWLETT, J. S.; HOFFMAN, G. C., and BATTLE, J. D., JR.: Radioactive isotopes in hematology. (Special exhibit.) Mod. Med. **28:** 87-93, March 1, 1960.

HIGGINS, C. C.: Blood in the urine demands immediate investigation. GP **21:** 109-111, March 1960.

HIGGINS, C. C.: The significance of blood in the urine. West Virginia M. J. **56:** 94-96, March 1960.

HOERR, S. O.: Management of acute cholecystitis—cholecystectomy or cholecystostomy? West Virginia M. J. **56:** 87-89, March 1960.

HOUSTON, C. S.;[4] AKUTSU, T., and KOLFF, W. J.: Pendulum type of artificial heart within the chest: preliminary report. Am. Heart J. **59:** 723-730, May 1960.

HURLEY, R. E.; PAGE, I. H., and DUSTAN, HARRIET P.: Bretylium tosylate as an antihypertensive drug. J. A. M. A. **172:** 2081-2083, April 30, 1960.

KARK, R. M.;[5] KOLFF, W. J.; POLLACK, V. E.,[5] and BEATTIE, E. J., JR.[5]: Symposium: Renal failure. Postgrad. Med. **27:** 512-521, April 1960.

KENDRICK, J. I.: Treatment of calcaneonavicular bar. J. A. M. A. **172:** 1242-1244, March 19, 1960.

KOLFF, W. J.: Artificial Organs: Heart; As Used For Heart Operations, pp. 33-36, *in* Medical Physics, Vol. 3, edited by O. Glasser. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

---

[4] *Formerly Research Associate; present address: Aspen, Colorado.*
[5] *University of Illinois College of Medicine, Chicago, Illinois.*

KOLFF, W. J.: Artificial Organs: Kidney; Past, Present and Future, pp. 36-39, *in* Medical Physics, Vol. 3, edited by O. Glasser. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

KOLFF, W. J.; EFFLER, D. B., and GROVES, L. K.: A review of four dreaded complications of open-heart operations; causes, avoidance, and treatment of acidosis, overoxygenation, heart-block, and pulmonary damage. Brit. M. J. 1: 1149-1153, April 16, 1960.

LEWIS, LENA A.: Electrophoresis, pp. 261-268, *in* Medical Physics, Vol. 3, edited by O. Glasser. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

McCULLAGH, E. P., and BRANDON, J. M.: Acromegaly. Chap. 4, pp. 36-47, *in* Clinical Endocrinology. I., edited by E. B. Astwood. New York: Grune & Stratton, Inc., 1960, 724 pp.

OMAE, T., and MASSON, G. M. C.: Compensatory adrenal hypertrophy as a possible cause of metacorticoid hypertension. Endocrinology **66**: 428-432, March 1960.

PAGE, I. H.: Arterial hypertension: its changing face. World-Wide Abs. Gen. Med. **2**: 9-14, Oct. 1959.

PAGE, I. H.: Newer antihypertensive drugs. Postgrad. Med. **27**: 448-454, April 1960.

PAGE, I. H., and BUMPUS, M.: Angiotensin: A Hormone of the Kidney. Chap. 6, pp. 591-597, *in* Clinical Endocrinology. I., edited by E. B. Astwood. New York: Grune & Stratton, Inc., 1960, 724 pp.

PROUDFIT, W. L., and DOBOSY, J. F.: Monitoring Devices: Electronic, pp. 391-394, *in* Medical Physics, Vol. 3, edited by O. Glasser. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

SCHOTZ, M. C., and RECKNAGEL, R. O.[6]: Effect of carbon tetrachloride on release of free fatty acids by rat adipose tissue. (25535). Proc. Soc. Exper. Biol. & Med. **103**: 398-400, Feb. 1960.

SINGER, S. J.;[7] FOTHERGILL, J. E.,[7] and SHAINOFF, J. R.: A new and general method for the isolation of antiprotein antibodies. J. Am. Chem. Soc. **82**: 565-571, Feb. 5, 1960.

TURNBULL, R. B., and GILL, NORMA: Accurate application of the ileostomy pouch; the use of a paper training sleeve. Ileostomy Quart. 4: 16-17, Spring 1960.

WASMUTH, C. E.: Editorial: The problem of *res ipsa loquitur*. Ann. Int. Med. **52**: 729-732, March 1960.

WASMUTH, C. E.: Legal pitfalls in the practice of anesthesiology; Part II: Complications of endotracheal anesthesia. Anesth. & Analg. **39**: 138-140, March-April 1960.

---

[6] *Department of Physiology, School of Medicine of Western Reserve University, Cleveland, Ohio.*
[7] *Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut.*

WASMUTH, C. E.: Liability of the anesthesiologist and the surgeon. J. A. M. A. 172: 1473-1475, April 2, 1960.

ZEITER, W. J.: Physical Medicine: Diathermy, p. 451, *in* Medical Physics, Vol. 3, edited by O. Glasser. Chicago: The Year Book Publishers, Inc., 1960, 754 pp.

THE FRANK E. BUNTS EDUCATIONAL INSTITUTE

affiliated with

THE CLEVELAND CLINIC FOUNDATION

announces the following postgraduate courses

Advances in Clinical Chemistry Methods . . . . . November 9, 10, and 11, 1960

Newer Developments in Ophthalmology . . . . . December 7 and 8, 1960

Upper Gastrointestinal Surgery . . . . . January 11 and 12, 1961

General Practice *(Sponsored by the Cleveland Chapter of the American Academy of General Practice)* . . . . . February 8 and 9, 1961

Peripheral Vascular Disease . . . . . March 1 and 2, 1961

Applications for registration are being accepted now.
Please communicate with

EDUCATION SECRETARY
THE FRANK E. BUNTS EDUCATIONAL INSTITUTE
2020 EAST 93 STREET
CLEVELAND 6, OHIO

Change of Address . . .

If you have changed your
permanent address, kindly
let us know on this form.

(Please type or print)

Name_____

Former address_____

_____

_____

New address_____

_____

_____

Cleveland Clinic Quarterly
2020 East 93 Street
Cleveland 6, Ohio